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(21)Application number : 04-158301 (71)Applicant : TAKEDA CHEM IND LTD

(22)Date of filing : 17.06.1992 (72)Inventor : IWASA SUSUMU
KUROKAWA
TOMOFUMI
TOYODA YUKIO

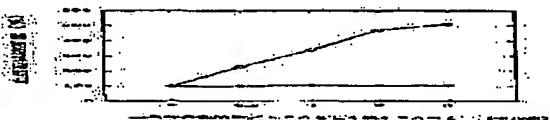
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(54) HYBRIDOMA-MONOClonAL ANTIBODY AND MEDICINE
CONTAINING ANTIBODY

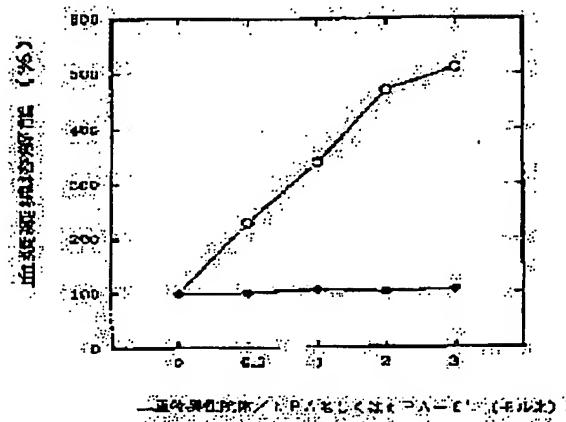
(57)Abstract:

PURPOSE: To prolong the half-life of
a tissue plasminogen activator(tPA)
as a fibrinolytic agent in blood, to



improve the lowering in the selectivity of tPA mutein for thrombus, and further to reduce the side effects of the fibrinolytic agent.

CONSTITUTION: The doubly specific monoclonal antibody in which one of the doubly specific properties relates to thrombus and the other also to tPA, e.g. tPA-6, lacking F, E and K1 domains. And, the fibrinolytic agent is produced by immunologically bonding the tPA mutein lacking the F, E and K1 domains to the doubly specific monoclonal antibody. Since the fibrinolytic ability and the selectivity for the thrombus are increased and further since the reactivity with fibrinogen is decreased, the fibrinolytic agent free from side effects such as the enhancement of the fibrinogen-decomposing ability is obtained.



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CLAIMS

[Claim(s)]

[Claim 1] The bispecific antibody whose one side of duplex singularity is a thing to organization plasminogen activator (tPA) MUTEIN to which another side comes to carry out deletion of a finger (F) domain, a growth factor (E) domain, and the (Kringle K) 1 domain to a thrombus.

[Claim 2] The bispecific antibody according to claim 1 whose thrombus is a fibrin.

[Claim 3] The bispecific antibody according to claim 1 whose thrombus is an activated platelet.

[Claim 4] The bispecific antibody according to claim 1 which contains a variable region at least and carries out deletion of the heavy chain constant region domains 2 and 3.

[Claim 5] The bispecific antibody according to claim 4 whose bispecific antibody is F(ab') 2.

[Claim 6] The thrombolytic agent which comes to carry out immunity association of tPA MUTEIN which comes to carry out deletion of the F, E, and K1 domain to a bispecific antibody according to claim 1.

[Claim 7] The thrombolytic agent according to claim 6 which is the thing to which tPA MUTEIN comes to carry out deletion of some thru/or all of a field of the amino-acid-residue numbers 296-302.

[Claim 8] The thrombolytic agent according to claim 6 which is the thing to which tPA MUTEIN comes to permute a part thru/or all of amino acid residue between the amino-acid-residue numbers 296-304 by another amino acid residue.

[Claim 9] The thrombolytic agent according to claim 7 which is the thing which tPA MUTEIN makes carry out deletion of some thru/or all of a field of the amino-acid-residue numbers 296-302, and comes to permute a part of amino acid residue of the field of the amino-acid-residue numbers 296-304 by another amino acid residue.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]**[0001]**

[Industrial Application] This invention relates to the hybrid monoclonal antibody (it may be hereafter written as MoAb) which has duplex singularity. One side of duplex singularity is related with the duplex singularity monoclonal antibody (it may be hereafter written as bsMoAb) which is a thing to tPA MUTEIN to which another side comes to carry out deletion of the F, E, and K1 domain to a thrombus in more detail. This invention relates to the thrombolytic agent which comes to carry out immunity association of tPA MUTEIN which comes to carry out deletion of the F, E, and K1 domain to above bsMoAb again.

[0002]

[Description of the Prior Art] A thrombolytic therapy is widely used for thrombus nature patients, such as myocardial infarction, arterial embolism, or cerebral infarction, and clinical application of streptokinase (it may be hereafter written as SK), the urokinase (it may be hereafter written as UK), etc. is carried out. Recently, the tissue plasminogen activator (it may be hereafter written as tPA) and prourokinase (it may be hereafter written as ProUK) which are said for the selectivity to a thrombus to be high, therefore to mitigate the side effect of a bleeding tendency tend to appear, and it is going to replace above-mentioned SK and UK. Furthermore, effectiveness is more high and the qualified type tPA etc. has been developed as a thrombolytic agent excellent in thrombus selectivity. moreover, the thrombolytic agent which used antibody targeting by one side -- appearing -- [C. -- Bode et al. -- :Science (Science) -- 229, 765;(1985) M.S.Runge et al.: Proceedings OBU National academy Science U.S.A.

(Proc.Natl.Acad.Sci.USA), 84 7659 (1987);T.Kurokawa et al. : [Biotechnology (Bio/Technology),] 7 and 1163 : (1989) The antibody which has high compatibility is used for the fibrin and activated platelet which form

0363712nd public presentation official report of the European Patent application referring-to-], and a thrombus. Compared with a thrombolysis active substance independent, a thrombolytic agent with high effectiveness also came to be produced dozens times from several times. Especially bsMoAb that can carry out immunity association at both thrombus and thrombolysis active substance offers the very efficient thrombolytic agent without the fall of antibody activity and thrombolysis activity.

[0003]

[Problem(s) to be Solved by the Invention] However, there being constraint that that large quantity must be prescribed for the patient over a long time since the half-life is very short, and often being accompanied by the side effect of a bleeding tendency also about tPA which appeared as a new generation's thrombolytic agent, for this reason is reported. For this reason, tPA MUTEIN of the type which does not receive inhibition by the inhibitor which exists in blood was produced in order to have made a half-life extend. However, since fibrin affinity ability is small, the selectivity to a thrombus falls, or as for such tPA MUTEIN, it turns out that the enzyme activity itself generally falls compared with the original unqualified type tPA. the side-effect [fibrinogen which a thrombolytic agent has in coincidence although it not only reinforces the thrombolysis ability of a thrombolysis active substance about an antibody targeting-ized thrombolytic agent again, but has the effectiveness of also making the half-life in the blood extending -- it turns out that increasing resolving-power and alpha2-antiplasmin (it being hereafter written as alpha2-AP) consumption ability] is not avoided to some extent.

[0004]

[Means for Solving the Problem] the fall of the thrombus affinity ability as which this invention persons were regarded by above-mentioned tPA MUTEIN -- or it found out extension of the half-life in blood, increase of thrombus affinity ability, and that mitigation of a side effect might be brought about further by repeating various examination, in order to solve the trouble of increase of the side effect seen by the antibody targeting-ized thrombolytic agent, making bsMoAb carry out immunity association of tPA MUTEIN, and carrying out targeting to a thrombus. That is, one side of duplex singularity is bsMoAb which is a thing to tPA MUTEIN to which another side carries out deletion of the F, E, and K1 domain to a thrombus, and this invention offers further the thrombolytic agent which makes this bsMoAb come to carry out immunity association of this tPA MUTEIN. The bispecific antibody of this invention can be combined with that whose thrombus is a fibrin or an activated platelet, and this antibody contains a variable region at least, and deletion of the heavy chain constant region domains 2 and 3 can be carried out.

[0005] Explanation of tPA MUTEIN constitutes tPA from FINGER (F), EGF (E), KRINGLE1 (K1) and KRINGLE2 (K2), and a PROTEASE (P) part, as shown in drawing 1 . tPA-6' of a publication etc. is mentioned as the below-mentioned example 19 of reference in which R298'299 of P part tPA-1 [given in the below-mentioned example 16 of reference in which E, F, and K1 part carried out deletion as above-mentioned tPA MUTEIN], and of them were permuted by E298'299. Moreover, an antibody molecule has an adjustable (V) field in connection with association with an antigen, and the stationary (C) field which participates in other biological functions, for example, association with complement, or association with an antibody receptor although it does not participate in antigen association, as shown in drawing 2 . When you need only the function in connection with antigen association, the molecule which consists of only variable regions is enough, and such a molecule is also produced in the latest gene recombination mold antibody. The approach adopted for many years is the approach of producing Fab or Fab' which presented restrictive partial hydrolysis processing with the antibody molecule using a digestive enzyme like a papain or a pepsin, cut the peptide in the part shown in drawing 2 , and carried out deletion of the (Pile H) chain constant region domains 2 and 3 (it may be written as CH2 and CH3 below, respectively). Thus, although the obtained antibody fragment loses some antibody functions, usually is held about 100% and antigen binding capacity at least can use such a fragment also in this invention.

[0006] As tPA MUTEIN used by this invention, deletion of all the domains of F, E, and K1 is carried out, and the further MUTEIN of K2, tPA MUTEIN (deletion of a part of 174-179 [the amino-acid-residue number 174-527 however], or all the amino acid residue may be carried out) which consists of both domains of a protease (P), and this tPA MUTEIN is mentioned (in accordance with both, it may be hereafter written as FEK1 deletion tPA MUTEIN). Moreover, originally as this further MUTEIN, that to which the original peptide or the proteinic amino acid sequence varied is mentioned, therefore the deficit of configuration amino acid and the permutation to other amino acid are mentioned as this variation. As a deficit of this configuration amino acid, that to which at least one of FEK1 deletion tPA MUTEIN configuration amino acid is missing is mentioned. As a permutation to these other amino acid, that by which at least one piece is permuted from another amino acid of FEK1 deletion tPA MUTEIN configuration amino acid is mentioned. Unless the description which FEK1 deletion tPA MUTEIN has is lost as the number of the configuration amino acid which is missing in FEK1 deletion tPA MUTEIN to which at least one configuration amino acid is missing, how many pieces are sufficient. As an example of this missing configuration amino acid, the amino acid residue of the amino-acid-residue numbers 296-302 of tPA etc. is mentioned.

[0007] As the number of the amino acid with which at least one configuration amino acid is permuted in FEK1 deletion tPA MUTEIN permuted from another amino acid, unless the description of FEK1 deletion tPA MUTEIN is lost, how many pieces are sufficient. As an amino acid part permuted, the thing which comes to permute a part thru/or all of amino acid residue between the amino-acid-residue numbers 296-304 by another amino acid residue, and the thing which is made to carry out deletion of some thru/or all of a field of the amino-acid-residue numbers 296-302, and comes to permute a part of amino acid residue of the field of the amino-acid-residue numbers 296-304 by another amino acid residue are mentioned as the example. As an example of the configuration amino acid before permuting, a cysteine, an aspartic acid, an arginine, etc. are mentioned, for example. When the configuration amino acid before permuting is a cysteine, as permuted amino acid, neutral amino acid is desirable, for example. As an example of this neutral amino acid, a glycine, a valine, an alanine, a leucine, an isoleucine, a thyrosin, a phenylalanine, a histidine, a tryptophan, a serine, threonine, a methionine, etc. are mentioned, for example. Especially, a serine and threonine are desirable.

[0008] When the configuration amino acid before permuting is things other than a cysteine, as permuted another amino acid, it is the point of the hydrophilic property of amino acid, hydrophobicity, or a charge, and a thing with a different property from the amino acid before permuting is chosen, for example. Although an asparagine, threonine, a valine, a phenylalanine, an arginine, etc. are mentioned as amino acid after permuting when the amino acid before specifically permuting is an aspartic acid, especially an asparagine and an arginine are desirable. Although a glutamine, glutamic acid, threonine, a leucine, a phenylalanine, an asparagine, and an aspartic acid are mentioned as amino acid after permuting when the amino acid before permuting is an arginine, especially glutamic acid or a histidine is desirable. When the amino acid before permuting is glutamic acid, a thyrosin is desirable as amino acid after permuting. That with which the above-mentioned deficit and the permutation combined is sufficient as MUTEIN of this invention.

[0009] In order to manufacture MUTEIN of this invention, in addition to the conventional recombinant DNA technology, a specific part directivity mutagenesis technique (Site-directed mutagenesis) is adopted. This technique is common knowledge and is shown to an R EFU racer (Lather, R.F.) and Jay Py REKOKKU (Lecoq, J.P.), JIENE tick engineering (Genetic Engineering), and the 31 - 50th pages (1983) of Academic Press. The mutagenesis directed to the oligonucleotide is em Smith (Smith, M.) and S GIRAMU (Gillam, S.), a JIENE tick engineering:principle and an approach, and three plenum press companies (1981). It is shown in 1 - 32 pages.

[0010] in order to manufacture the structural gene which carries out the

code of MUTEIN of this invention, a primer is expanded for example, by (1) (2) which carries out hybridization of single stranded DNA which consists of single strand of structural gene of tPA to mutation oligonucleotide primer DNA polymerase, and a mutation heterodimer (heteroduplex) is made to form -- (3) -- there is a method of reproducing this mutation heterodimer. [and]

[0011] Which thing may be used as long as this hybridoma produces to a fibrin MoAb which it is specific and is not substantially combined with a fibrinogen in production of the anti-fibrin antibody production hybridoma used by this invention. [K.Y.Hui et al. [for example,] produced when an alpha-chain amino-terminal fragmentation peptide or beta-chain amino-terminal fragmentation peptide of the fibrin which a fibrinogen is disassembled and is produced is used for this fibrin specific antibody as immunogen: They are Science (Science), 222, and 1129(1983); JP,63-93800,A]. Moreover, although any are sufficient as long as a fibrin is the thing of mammalian, a Homo sapiens fibrin is mentioned preferably and the peptide which is especially equivalent to the beta chain amino terminal section of a Homo sapiens fibrin is used. Carriea protein is combined with this, immunity of the animals (an example, a rabbit, a rat, a mouse, guinea pig, etc.) is carried out, and an antibody forming cell is obtained. Subsequently, the antibody forming cell which unites with a myeloma cell these antibody forming cells extracted from the immune animal, for example, a spleen cell, a lymph gland cell, etc., does not react to a fibrinogen substantially but is specifically combined with a fibrin out of the hybridoma obtained is screened. As the above-mentioned Homo sapiens fibrin beta chain amino terminal peptide, what has an amino acid sequence like a degree is used especially preferably.

H-Gly-His-Arg-Pro-Leu-Asp-Lys-R-Cys-OH [[R shows among a formula the peptide shown by Lys-Arg-Glu-Glu, or its part] the array number 1]. Cys of a C terminal is used for the linker section for chemical bonds with carriea protein. That is, it is possible to carry out the chemical bond of the carriea protein to carriea protein through the sulphydryl group of C terminal Cys of the above-mentioned peptide beforehand by dithiopyridyl-izing by maleimide-izing or N-*SAKUSHINIMIJIRU-3-(2-pyridyl dithio) propionate* (it being hereafter written as SPDP) for example, by N-(gamma-maleimidebutyloxy SAKUSHINIMIDO) (it being hereafter written as GMBS).

[0012] In order to make fibrin singularity into a positive thing furthermore, using the copolymer (it may be hereafter written as D-GL) of D-glutamic acid and D-lysine which T.Hamaoka and others reported, a fibrinogen tolerant condition is made to cause in a fibrin antigen immune animal, and the method of obtaining a fibrin specific antibody production cell by high frequency more

is also used preferably. [— T — a .Hamaoka;journal OBU EKUSUPURI mental medicine (139, 1446(1974);K.Tateishi et al. [J.Exp.Med.],] : journal OBU immuno logical MESOZZU (J.Immunol.Methods), 47, 249] (1981.) By this approach, the thing which what combined carries protein with the above-mentioned Homo sapiens fibrin beta chain amino terminal peptide as immunogen made combine D-GL with the peptide which has an amino acid sequence like a degree as tolerogen is used.

H-Phe-Phe-Ser-Ala-Arg-Gly-His-Arg-Pro-Leu-Asp-Lys-R-OH [[the peptide shown by Lys-Arg-Glu-Glu or its part is shown by the inside R of a formula] the array number 2].

[0013] Moreover, which thing may be used as long as this hybridoma produces MoAb which is not combined with a non-stimulating platelet specifically substantially at an activated platelet in production of the anti-activated platelet MoAb production hybridoma used by this invention. This activated platelet unique MoAb for example, by using as immunogen the platelet (preferably Homo sapiens platelet) activated by the thrombin [C.L.Berman et al. produced : A journal OBU clinical investigation (J.Clin.Invest.), 78, 130;(1986) N.Akamatsu et al.: SURON baud cis- - and - HEMOSUTASHISU (Thromb.Haemostasis), 62, 250 (1989)]. Moreover, although any are sufficient as long as the platelet to be used is the thing of mammalian, a Homo sapiens platelet is mentioned preferably. Immunity of this activated platelet is carried out to animals (an example, a rabbit, a rat, a mouse, guinea pig, etc.), an antibody forming cell is obtained, and these antibody forming cells subsequently extracted from the immune animal, for example, a spleen cell, a lymph gland cell, etc. are united with a myeloma cell. By screening the antibody forming cell which does not react to a non-stimulating platelet substantially but is specifically combined with an activated platelet out of the hybridoma obtained, the anti-activated platelet MoAb production hybridoma made into the purpose is acquirable. About production of the anti-tPA MUTEIN MoAb production hybridoma in this invention, immunity of tPA MUTEIN mentioned above or the tPA of the original unqualified type itself is carried out to an animal according to a conventional method, and the method of uniting the antibody forming cell obtained with a myeloma cell etc. is used further again. Immunity of the animal is carried out, the antibody forming cell obtained is united with a myeloma cell etc., and the same actuation as the approach of obtaining an anti-fibrin antibody production hybridoma or an anti-activation platelet antibody production hybridoma is used about the approach of obtaining an antibody production hybridoma.

[0014] As an immune animal, although a rabbit, a rat, a mouse, a guinea pig, etc. are used, for example, in MoAb manufacture, a mouse is used especially preferably. In producing a specific antibody to an activated platelet, to a

mouse that what is necessary is just to follow the approach usually enforced as the inoculation approach 10⁸–10¹⁰ per time 0.5 to 2×10⁹ washing Homo sapiens platelets are preferably suspended in a physiological saline, the HEPESU buffer solution, or the phosphoric-acid salt buffer solution (it may be hereafter written as PBS), and the approach of inoculating into day by day [10 – 14] 3 to 8 times in an activation postabdomen cavity by the thrombin is taken. When producing a Homo sapiens fibrin and an antibody specific to tPA MUTEIN, preferably, the antigenic proteins of 10–25microg are emulsified in the physiological saline of the amount (0.1ml) of isochore, and the perfect AJU band of Freund, and 1 time 1–100microg and the approach of inoculating into intraperitoneal [of regions of back and an abdomen / hypodermically or intraperitoneal] 3 to 6 times every 2–3 weeks are taken by the mouse. an individual with these immune animals, for example, a mouse, to high antibody titer -- choosing -- three – five days after the last immunity -- a spleen -- and -- or lymph gland is extracted and the antibody forming cell contained in them is united with a myeloma cell. Although fusion actuation can be carried out according to a known approach and a polyethylene glycol (it may be hereafter written as PEG), an Sendai virus, etc. are mentioned as a fusion accelerator, PEG is used preferably. Especially as a myeloma cell, NS-1 and P3U1 are preferably used for NS-1, P3U1, SP2/0, etc. For example, the desirable ratios of a spleen cell and a myeloma cell are 1:1–10:1, and are good for PEG of molecular weight 1,000–9,000 to be added by this by 10 – 80% of concentration, and for 20–37 degrees C to incubate at 30–37 degrees C preferably for 3 to 10 minutes. [0015] Various approaches can be used for screening of an anti-fibrin antibody production hybridoma. For example, a thrombin is made to act and a fibrinogen is changed into a fibrin, after making a fibrinogen stick to a microplate. Subsequently, a hybridoma culture supernatant is added to a fibrin fixed microplate under a superfluous fibrinogen existence, and the antibody titer in a culture supernatant is measured with the enzyme immunoassay (it may be hereafter written as EIA) which detects the anti-fibrin specific antibody combined with the plate. Although cloning is immediately presented with the hybridoma of the sorting and the antibody activity positivity by which the breeding was carried out in a HAT (hypoxanthine aminopterin thymidine) addition culture medium, this is usually easily carried out by limiting dilution etc. The antibody titer of the cloned hybridoma culture supernatant can be measured by the above-mentioned approach, the hybridoma which produces an antibody with a stably high potency can be chosen, and the monoclonal anti-fibrin specific antibody production hybridoma made into the purpose can be acquired. According to the above manufacturing methods, FIB 1–11 and FIB 2–11 which were shown in the below-mentioned example 8 of reference and a below-mentioned

example 1, and FTB 2-133 are mentioned as an example of the produced anti-fibrin antibody production hybridoma. Various approaches can be used also about screening of an anti-activated platelet MoAb production hybridoma. For example, a non-stimulating platelet or a thrombin activated platelet is combined with a microplate, and it fixes with formalin 1%, and is used as a solid phase antigen. A hybridoma culture supernatant is added to this, the antibody titer in a culture supernatant is measured by EIA which detects the anti-activation platelet antibody combined with the plate by the 2nd antibody of enzyme labeling, and the big thing of the difference of un-stimulating and association with an activated platelet is chosen. For example, although cloning is immediately presented with the hybridoma of the sorting and the antibody activity positivity by which the breeding was carried out in a HAT addition culture medium, this is usually easily carried out by limiting dilution etc. The antibody titer of the cloned hybridoma culture supernatant can be measured by the above-mentioned approach, the hybridoma which produces an antibody with a stably high potency can be chosen, and the monoclonal anti-activated platelet specific antibody production hybridoma made into the purpose can be acquired. Mouse hybridoma 2T60 shown in the below-mentioned example 9 of reference as an example of the anti-activated platelet MoAb production hybridoma produced according to the above manufacturing methods are mentioned.

[0016] Moreover, screening of the hybridoma which produces MoAb to tPA MUTEIN can be carried out simple in EIA using the microplate to which tPA MUTEIN or an unqualified type tPA was made to stick. It carries out according to the conventional method which also described cloning above, and the target anti-tPA MUTEIN MoAb production hybridoma can be acquired. The mouse hybridoma TPA 1-41 shown in the below-mentioned example 10 of reference as an example of the anti-tPA MUTEIN MoAb production hybridoma produced according to the above manufacturing methods is mentioned.

[0017] Although there is some technique in production of the poly dahoma which produces the hybrid MoAb which has the duplex singularity of this invention and [which [an example, Yoji Niimoto:protein, a nucleic acid and an enzyme, 33, 217] (1988), etc. and] approach may be used, for example ** Make the culture medium of 5-bromodeoxyuridine (it may be hereafter written as BrdU) addition acclimate gradually the anti-tPA MUTEIN antibody production hybridoma of the above-mentioned HAT resistance, clone a thymidine kinase deficit stock, and consider as HAT susceptibility. The anti-fibrin of HAT resistance or an anti-activated platelet specific antibody production hybridoma is similarly made into 8-azaguanine (it may be hereafter written as AZG) resistance, and a hypoxanthine-guanine-phosphoribosyl-transferase deficit stock is cloned,

and it considers as HAT susceptibility. Tetra-OMA obtained by uniting both according to a conventional method by the HAT addition culture medium. Subsequently, after sorting, Clone tetra-OMA which secretes the hybrid MoAb which has a binding affinity in both thrombus and tPA MUTEIN. An anti-fibrin or an anti-activated platelet specific antibody production hybridoma ** A fluorescein isothiocyanate An indicator is carried out with (it may be hereafter written as FITC), and both are united for another anti-tPA MUTEIN antibody production hybridoma after an indicator according to a conventional method by tetramethyl RODAMIN isothiocyanate (it may be hereafter written as TRITC). A fluorescein bitter taste tee bay TIDDO cell sorter (it may be hereafter written as FACS) is presented with the obtained cell suspension, and approaches, such as sorting out and cloning tetra-OMA which has the fluorescence of the green of FITC and the red of TRITC in coincidence, are mentioned. Moreover, the marker of a parents stock is completely used, making it reverse, from the mouse which carried out immunity of possible **tPA MUTEIN, a spleen cell is extracted, the anti-fibrin or anti-activated platelet MoAb production hybridoma made into HAT susceptibility by one side, and this spleen cell are united, it sorts out by the HAT addition culture medium, and sorting out and cloning tetra-OMA also creates TORIOMA. Subsequently, the approach of cloning TORIOMA which secretes the hybrid MoAb which has a binding affinity is in both thrombus and tPA MUTEIN.

[0018] if in charge of the cell fusion in these actuation -- fusion accelerators, such as Sendai Virus and PEG, -- or approaches, such as electrical stimulation, are used. Although PEG is used preferably and that example is given to below, of course, it is not limited to this approach. That is, PEG(s), such as about 10 – 80% of molecular weight about 1,000 to 9,000 concentration etc., are used, and although the processing times are about 0.5 – 30 minutes, as an example of desirable conditions, for about 4 – 10 minutes, about 35 – 55% of PEG6,000 can be contacted into a cell, and can be efficiently united at 37 degrees C. Although selection of poly dahoma can be carried out by the above-mentioned HAT addition culture medium etc., for this reason, each drug tolerance stock is acquired by the drugs acclimating methods, such as 8-AZG, 6-thioguanine (6-TG), or 5-BrdU. Moreover, various selective media are used by installation to the new syncytium of a marker. As such an example, a neomycin, a hygromycin B addition culture medium, etc. are mentioned [B.Sugden:molecular – and – cellular biology (Mol.Cell.Biol.), 5, and 410 (1985)]. the [L.Karawajew:journal OBU immuno which the approach of carrying out sorting of the hybrid hybridoma by which united the hybridoma which furthermore carried out the indicator by said different fluorochrome as carried out, and the double labelling was carried out by FACS also has -- logical – MESOZZU (J. Immunol.Methods) .96 and

265 (1987)].

[0019] Various approaches can be used for screening of hybrid antibody production poly dahoma. ** above-mentioned For example, the EIA concomitant use for screening of the anti-fibrin or anti-activated platelet specific antibody production hybridoma carried out, and an anti-tPA MUTEIN antibody production hybridoma, ** Add *****-ed to fibrin association or an activated platelet joint microplate. Next, when using the antibody to tPA MUTEIN belonging to a different subclass from EIA; for the hybrid antibody detection which adds tPA MUTEIN or the unqualified type tPA which carried out the HRP indicator, and has duplex singularity, an anti-fibrin, or an anti-activated platelet specific antibody ** It can use, combining suitably EIA which adds *****-ed to fibrin association or an activated platelet joint microplate, adds this anti-mouse IgG subclass specific antibody that carried out the HRP indicator next, and detects a bispecific antibody, the strange method of these, etc.. Although cloning is immediately presented with the poly dahoma of a hybrid antibody activity positivity, this is usually easily carried out by limiting dilution etc. About the culture supernatant of the cloned poly dahoma, the monoclonal hybrid antibody production poly dahoma made into the purpose is acquirable by measuring the antibody titer by the above-mentioned approach, and choosing the poly dahoma which produces an antibody with a stably high potency.

[0020] Culture of the poly dahoma of above-mentioned this invention can usually be carried out by the well-known approach by intraperitoneal [of an animal] (for example, intraperitoneal [of mammals, such as a mouse,]) among a liquid medium. It can do by using combining a well-known biochemical model about purification of culture medium and an antinode underwater antibody. For example, centrifugal separation of cell culture liquid or the ascites is carried out, supernatant liquid is taken out, and a salting-out (an ammonium sulfate or a sodium sulfate is usually used) is carried out. The obtained protein precipitate can be dissolved in a suitable solution, the column chromatographies after dialysis (an ion exchange column, a gel-filtration column, a protein A column, a hydronalium KINAPA tight column, an ABx column, a canal support column, or antigen joint column) can be given, and separation purification of the target antibody can be carried out. By the above separation purification actuation, about 1-5mg of hybrids MoAb of 90% or more of purity can be obtained from a 1l. culture supernatant by the protein weight ratio. Moreover, 3-10mg of same antibodies is obtained from 20ml antinode water. TAF 1-42 which FT 2-14 shown in the example 3 as an example of anti-fibrin-anti-tPA MUTEIN bispecific antibody production tetra-OMA produced according to the above manufacturing methods showed to the example 2 as an example of TORIOMA, TAF 1-79, and TAF 1-228 are mentioned. When the bispecific antibody of this invention is the thing of the

mouse antibody origin The fields, for example, the constant region, or framework regions other than the hypervariable region of this protein a genetic manipulation technique -- the thing of the *Homo sapiens* antibody origin -- changing -- [Z. Steplewski et al. -- the :pro C DINGUZU OBU National academy Science U.S.A. (Proc.Natl.Acad.Sci.U.S.A) -- 85 4852; (1988) L.Riechmann et al.: Nature (Nature), 332,323 (1988)], a mouse-*Homo sapiens* chimera mold, or the antibody that carried out ize [HYUMA] is also producible. On the occasion of administration to *Homo sapiens*, since antigenic is small, this *Homo sapiens* mold-sized antibody is used advantageously.

[0021] Although the above-mentioned hybrid hybridoma method is desirable in order to produce the bispecific antibody of this invention, the following chemical bond methods are also used. In order to combine two sorts of MoAb(s) chemically, the substituent which exists in an antibody molecule, for example, the amino group, a carboxyl group, hydroxyl, or a sulfhydryl group can be used. The reactant amino group of (1) one antibody, and the reactant carboxyl group of another side For example, the example of water-soluble carbodiimide reagent [, 1-ethyl -3 -(3-dimethylaminopropyl)- Carbodiimide, 1-cyclohexyl -3 -(2-morpholino ethyl)- Carry out dehydration condensation in an aquosity solvent using], such as carbodiimide-p-toluene sulfonate. (2) The reactant amino group of an antibody On the other hand, the example of activity ester [of N-hydroxy SUKUSHIMIDO, p - Maleimide methylcyclohexane-1-carboxyl-N-hydroxy SUKUSHIMIDO ester, After making it react with], such as N-(epsilon-maleimide KAPURO yloxy) SUKUSHIMIDO ester or GMBS, and maleimide-izing, i) The antibody which returned the antibody of another side by dithiothreitol (DTT), or the antibody which introduced the sulfhydryl group into the antibody of ii another side by N-SUKUSHIMIJIRO-3-(2-pyridyl dithio) propionate (SPDP), Or carry out thioether association of the antibody of iii another side with the sulfhydryl group of the Fab' fraction obtained by carrying out pepsin digestion post reduction. (3) Combine two sorts of reactant amino groups of both antibody using dialdehyde reagents, such as succindialdehyde and glutaraldehyde. (4) It reoxidates, after introducing two sorts of antibodies by DTT, introducing a sulfhydryl group by reduction or SPDP, carrying out the pepsin digestion post reduction of each (5) 2 sort antibody which produces a heterodimer by reoxidation and considering as Fab', and there is the approach of producing a Fab' heterodimer. moreover, the ** which does not spoil two sorts of antibody activity as much as possible combining various these approaches -- efficient -- target hetero DAIME -- there is a report which produces a rucksack bispecific antibody and it can use for production of the duplex singularity MoAb of [M.J.Glennie:journal OBU immunology (J. Immunol.), 139, 2367:(1987) Kitagawa Tsunehiro:synthetic organic chemistry, 42 and 283

(1984)] and this invention.

[0022] A bispecific antibody connective can be refined and isolated preparatively after the above ligation reaction termination with gel filtration chromatography, such as sephadex G100 or G200, sepharose 6B or 4B, URUTOROGERU AcA44 or 34, and sephacryl S200. Or an alternative aliquot is also possible by combining the affinity chromatography using an antigen joint column.

[0023] The hybrid MoAb which has the duplex singularity acquired as mentioned above is uniform as protein, and can obtain F(ab')2 fragment holding an activated platelet or a fibrin, and the binding affinity to tPA MUTEIN etc. by proteolytic enzyme processings (a pepsin, papain, etc.) etc. Some approaches are used in the thrombolytic treatment method using the alternative thrombolysis protein complex produced from the duplex singularity MoAb of this invention, or tPA MUTEIN and this duplex singularity MoAb. for example, BE ** combined with the thrombus which medicated the thrombus nature disease patient with the duplex singularity MoAb of ** this invention beforehand, and was formed in the patient inside of the body -- a thrombus nature disease patient is medicated with the ** this duplex singularity MoAb and tPA MUTEIN which prescribe tPA MUTEIN for the patient after sufficient time amount progress at coincidence. or ** -- this duplex singularity MoAb and tPA MUTEIN are made to react beforehand, for example, immunity association is carried out, and the approach of medicating a thrombus nature disease patient with the obtained alternative thrombolysis protein complex and alternative thrombolysis protein complex [as / whose ratio of this bSMoAb and tPA MUTEIN is 1:1 preferably] after separating unreacted tPA MUTEIN is mentioned.

[0024] The duplex singularity MoAb or the thrombolytic agent which comes to carry out immunity association of tPA MUTEIN further of this invention After the filtration disinfection actuation by a membrane filter etc., mix with itself or the proper support which may be permitted in pharmacology, an excipient, a diluent, etc. as occasion demands, and it pharmaceutical-preparation-izes as injections etc. It is possible to medicate mammalians (an example, a mouse, a rat, a cat, a dog, Buta, a cow, an ape, Homo sapiens, etc.), for example, to use for the therapy of a thrombus and obstructive diseases, such as myocardial infarction, ***** and the vein obstruction, ***** and the vein obstruction, cerebral infarction, and pulmonary embolism. Generally about 0.004 to 0.04 mg/kg is preferably desirable [the dose of the thrombolytic agent of this invention / per day / in about 0.04 to 0.4 mg/kg, and tPA MUTEIN] as duplex singularity MoAb, when administering intravenously to the adult patient of myocardial infarction, for example, although it changes with the target disease, a symptom, or administration roots also in about 0.002 to 0.1 mg/kg about 0.001 to 0.5

mg/kg preferably about 0.02 to 1 mg/kg.

[0025]

[Function] By the above approaches, it is specifically combinable to a target thrombus part, and a thrombus can be dissolved and removed alternatively and efficiently by using the duplex singularity MoAb and tPA MUTEIN of this invention which are substantially combined neither with a fibrinogen nor a non-stimulating platelet, without being accompanied by the side effect.

[0026] In this invention specification and a drawing, when displaying a base, amino acid, etc. by the cable address, the following of the example is carried out based on the cable address by IUPAC-IUB Commision on Biochemical Nomenclature, or the common use cable address in the field concerned.

Moreover, especially when there may be an optical isomer about amino acid, L-object shall be shown if not shown clearly.

DNA : Deoxyribonucleic-acid cDNA : Complementary deoxyribonucleic acid A : Adenine T : Thymine G : Guanine C : Cytosine RNA : Ribonucleic-acid dATP : Deoxyadenosine triphosphoric acid dTTP : Deoxythymidine triphosphoric acid dGTP : Deoxyguanosine triphosphoric acid dCTP : Deoxycytidine triphosphoric acid ATP : An adenosine triphosphate Tdr : Thymidine EDTA : Ethylenediaminetetraacetic acid SDS : Sodium dodecyl sulfate Gly (G) : Glycine Ala (A) : Alanine Val (V) : Valine Leu (L) : Leucine Ile (I) : Isoleucine Ser (S) : Serine Thr (T) : Threonine Cys (C) : Cysteine Met (M) : Methionine Glu (E) : Glutamic acid Asp (D) : An aspartic acid Lys (K) : Lysine Arg (R) : Arginine His (H) : Histidine Phe (F) : Phenylalanine Tyr (Y) : Tyrosine Trp (W) : Tryptophan Pro (P) : Proline Asn (N) : Asparagine Gln (Q) : Glutamine.

[0027]

[Example] Although the example of reference and an example explain this invention concretely below, it cannot be overemphasized that it is not that to which these restrict the range of this invention. In addition, deposition is performed as the animal cell and microorganism which are used in the example of reference and the example are shown in the following tables.

(IFO) (FRI) An animal cell and a microorganism name IFO No. FERM No. A mouse hybridoma 50174 BP-2081 FIB 1-11 A mouse (1988. 10.4) hybridoma (1988. 9.21) 50175 BP-2082 FIB 2-11 A mouse hybridoma (1988. 9.21) (1988. 10.4) 50178 BP-2085 TPA 1-41 (1988. 9.21) Mouse (1988. 10.4) hybridoma 50211 BP-2623 2T60 The mouse (1989. 10.4) hybridoma 50332 (1989. 9.27) BP-3455 FTB 2-133 (1991. 6.7) (1991. 6.18) Mouse hybrid hybridoma 50180 BP-2158(tetra-OMA) FT 2-14 (1988. 11.8) (1988. 11.25) Mouse hybrid hybridoma 50333 BP-3456(TORIOMA) TAF 1-42 (1991. 6.7) Mouse hybrid hybridoma (1991. 6.18) 50334 BP-3457(TORIOMA) TAF 1-79 (1991. 6.7) (1991. 6.18) Mouse hybrid hybridoma 50335 BP-3458(TORIOMA) TAF 1-228 (1991. 6.7) (1991. 6.18) Escherichia coli MM294 (DE3) / pLysS, pTB1133 IFO 15031 FERMBP-2882 Escherichia coli MM(1990. 5.1)294 (DE3) / pLysS,

pTB1277 IFO 15116 FERM BP-3199 (1990. 4.17) (1990. 12.6) IFO (1990. 12.13): Institute for Fermentation, Osaka (2-17-85, Jusohommachi, Yodogawa-ku, Osaka-shi)

FRI: The Fermentation Research Institute, the Ministry of International Trade and Industry, (1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken)
[0028]

[The example 1 of reference] EIA3.3M urea for anti-fibrin antibody measurement, 0.01% The 1mg [/ml] Homo sapiens fibrin monomer solution which dissolved in the EDTA content phosphoric-acid salt buffer solution (PBS, pH7.3) was poured distributively every [50micro / 1] to 96 hole microplate, after [overnight neglect], 2% casein, and 0.01% thimerosal content PBS150microl was added at 4 degrees C, and the sensitization plate was produced. Next, 100 units / ml heparin, and the 10mg [/ml] Homo sapiens fibrinogen solution that dissolved in 3mM phenylmethyl sulfonyl fluoride content PBS are mixed with an equivalent **-ed hybridoma culture supernatant, and the 100microl was added on the above-mentioned fibrin sensitization plate after the reaction for 30 minutes at the room temperature, and it was made to react at a room temperature for 2 hours. The horse RADDISSHU peroxidase (HRP) indicator rabbit anti-mouse IgG antibody was fully added for the plate after washing by the Tween20 content PBS (PBS-Tw) 0.05%, and it was made to react at a room temperature further for 2 hours. The 0.1M citrate buffer solution which contains an orthochromatic phenylenediamine and H2O2 as an enzyme substrate was added to each well after washing, and the enzyme reaction was carried out at the room temperature. The amount of coloring coloring matter was measured on the wavelength of 492nm after the reaction halt using the multi-scan (flow company make) with 1-N sulfuric acid.

[0029]

[The example 2 of reference] Platelet rich plasma was acquired from the Homo sapiens fresh blood in which the EIA** fixed platelet for antiplatelet antibody measurement carried out production citric-acid blood collecting with the centrifuge method, and the tie load-HEPESU buffer solution (pH6.5) containing an ADP dialytic ferment washed. this washing platelet was scattered to the microplate by 2x107 pieces / well, and it was activated by the thrombin (0.2 units / ml) -- it carried out after centrifugal. Subsequently, it blocked with formalin 5% after immobilization 2% by the cow serum albumin (it may be hereafter written as BSA) content PBS, and the activated platelet plate was produced. The non-stimulating platelet plate omitted and produced thrombin activation actuation during the above-mentioned actuation.

** 100micro of EIA operation information hybridoma culture supernatants I was added on the platelet plate, washing and a HRP indicator rabbit anti-mouse IgG antibody were added by PBS-Tw after a 3-hour reaction at

the room temperature, and it was made to react at a room temperature further for 2 hours. It measured after washing by the approach which showed the HRP activity combined with solid phase to the example 1 of reference.

[0030]

[The example 3 of reference] The single-strand tPA(central science industrial KK. sale)5microg/ml solution of EIA marketing for anti-tPA antibody measurement was poured distributively every [100micro / 1] to 96 hole microplate, after [overnight neglect], 2% casein, and 0.01% thimerosal content PBS150microl was added at 4 degrees C, and the sensitization plate was produced. The above-mentioned liquid is removed, and 100micro of **-ed hybridoma culture supernatants I was added after washing by PBS-Tw, and it was made to react at a room temperature for 2 hours. Hereafter, the enzyme reaction was carried out by the approach of a publication for the example 1 of reference, and antibody titer was measured.

[0031]

[The example 4 of reference] tPA-6' given in the example 19 of reference was used instead of tPA given in the example 3 for anti-tPA MUTEIN antibody measurement of EIA reference, and anti-tPA MUTEIN antibody titer was measured by the same approach after producing a tPA-6' sensitization plate.

[0032]

[The example 5 of reference] The commercial Homo sapiens fibrinogen (Wako Pure Chem sale) was used instead of tPA given in the example 3 for anti-Homo sapiens fibrinogen antibody measurement of EIA reference, and anti-Homo sapiens fibrinogen antibody activity was measured by the same approach after producing a Homo sapiens fibrinogen sensitization plate.

[0033]

[The example 6 of reference] anti- -- the hybrid antibody content test liquid was added on the tPA-6' sensitization plate created in the example 4 for fibrin-anti-tPA MUTEIN hybrid antibody measurement of EIA reference, and it was made to react to it at a room temperature for 2 hours. The Homo sapiens fibrin beta chain amino terminal peptide (1-11)-BSA complex of a publication was added to example of reference 8-** which carried out the biotin indicator after washing by PBS-Tw, and it was made to react to it at a room temperature further for 2 hours. Next, it measured by the approach which showed the HRP activity which added avidin-HRP complex and was combined with solid phase after the 1-hour reaction at the room temperature to the example 1 of reference.

[0034]

[The example 7 of reference] The **-ed hybridoma culture supernatant diluent was added in the fibrinolysis protection-test tPA solution (the 20 ng/ml last concentration), and 5microl impregnation per one well of a fibrin

agarose plate of the cocktail after a 1-hour reaction was done at 37 degrees C. The dissolution spots (diameter) of a fibrin were measured 2 – 6 hours after at 37 degrees C, and the neutralization ability of MoAb contained in the hybridoma culture supernatant to the enzyme activity of tPA was measured. [0035]

[The example 8 of reference] preparation of the production ** immunogen of a mouse anti-Homo sapiens fibrin monoclonal antibody production hybridoma -- a well-known solid phase synthesis method -- a peptide synthesis machine (an applied system --) Homo sapiens fibrin beta chain amino terminal peptide (1-11)-Cys 3.3mg of the degree type produced using the model 430A mold It was made to react at 30 degrees C for 1 hour in addition to the BSA(for 13 mols [per one mol of BSAs] maleimide radical to be introduced)12mg/2ml water solution beforehand maleimide-ized by GMBS, and Homo sapiens fibrin beta chain amino terminal peptide (1-11)-BSA complex was obtained. Subsequently, with the physiological saline, after 3 times dialysis (3 l. x3), cryopreservation was carried out and it used as immunogen.

The equivalent Freund perfect AJU band was added to the H-Gly-His-Arg-Pro-Leu-Asp-Lys-Lys-Arg-Glu-Glu-Cys-OH ** (12-mer, array number 1) immunity peptide-BSA 1mg [/ml] complex physiological saline solution, and the regions of back of a mouse (**, n=10:0.1mg / 0.2ml / mouse) and the immunity of an abdomen hypodermically were started. the Freund's uncomplete adjuvant of equivalence [booster / immunogen] -- in addition, it inoculated 5 times and carried out every two to three weeks.

** The spleen was extracted in three days after the cell fusion last immunity, and spleen cell suspension was prepared with the conventional method (about 108 pieces). Subsequently, 2x10⁷ mouse myeloma cells (P3U1) were added, and cell fusion was presented according to the approach [Nature (Nature) .256,495 (1975)] of Kohler and Milstein using PEG6000. After fusion termination, cell mixture was suspended in the HAT medium and cultivated for ten days. Henceforth, as soon as selection of a parent cell was completed, henceforth it replaced with HT culture medium excluding aminopterin from the HAT medium, and culture was continued.

[0036] ** The antibody titer of a hybridoma culture supernatant was measured in EIA of a publication for the examples 5 and 1 of reference using the microplate which made the Homo sapiens fibrinogen and the Homo sapiens fibrin monomer stick to selection and the cloning solid phase of a hybridoma. The antibody which will accept the appearance of a hybridoma after 20 days from fusion the 10th, and carries out unique association at a Homo sapiens fibrin was seen. Cloning by limiting dilution was presented about the strong hybridoma of especially avidity. Screening of EIA was similarly presented with the culture supernatant of the hybridoma which

cloned, and the strong thing of a *Homo sapiens* fibrin binding affinity was chosen. The MoAb production mouse hybridomas [FIB / FIB and / 2-11] 1-11 which carry out unique association were obtained by the fibrin under high concentration *Homo sapiens* fibrinogen existence these results. The immunoglobulin class of MoAb FIB 1-11 obtained from these and FIB 2-11 and the subclass were measurement by the Ouchterlony technique, and were all IgG1 (kappa chain).

[0037]

[The example 9 of reference] The washing platelet was acquired from the *Homo sapiens* fresh blood obtained by production ** immunity citric-acid blood collecting of a mouse anti-activation platelet antibody production hybridoma with the centrifuge method. After adding thrombin 0.1 unit / ml to about 109 platelets and incubating for 5 minutes at 37 degrees C, it injected intraperitoneal [of a BALB/c mouse] (n= 10; about 109 pieces / 1ml / mouse). 6 to 8 times immunity was carried out every two weeks.

** NS-1 was used for example of cell fusion reference 8-** instead of mouse myeloma cell P3U1 of a publication, and cell fusion was completely carried out by the same approach below.

** The hybridoma was screened in EIA selection of a hybridoma, and given in the example 2 of reference using a cloning platelet joint microplate, and the anti-activated platelet MoAb production hybridoma was acquired in the same way as example of reference 8-** below. MoAb production hybridoma 2T60 specifically combined with *Homo sapiens* and a rabbit activated platelet were obtained these results. The immunoglobulin class of antibody 2T60 and the subclass were measurement by the Ouchterlony technique, and were IgG1 (kappa chain).

[0038]

[The example 10 of reference] the Freund perfect AJU band of equivalence [solution / of a mouse anti-tPA MUTEIN monoclonal antibody production hybridoma / production ** immunity tPA200microg/ml physiological saline] -- adding -- enough -- after emulsion and a BALB/c mouse (**, 20micro g/0.2ml / mouse) -- the abdominal cavity -- and regions-of-back hypodermic administration was carried out, and the booster was carried out at intervals of 2-3 weeks. tPA antigen liquid (50micro g/0.1ml physiological saline / mouse) was administered intravenously after 3 times of boosters about the individual which showed the blood serum antibody titer greatest in ten days.

** According to the approach of a publication, cell fusion was carried out to example of cell fusion reference 8-**.

** The anti-tPA MoAb production hybridoma which screens a hybridoma in EIA selection of a hybridoma and given in the example 4 of reference using a cloning tPA MUTEIN joint microplate, and has tPA MUTEIN avidity by the

same approach as example of reference 8-** below was acquired. Anti-tPA which does not show neutralization ability in these to the example 7 of reference by the fibrinolysis protection test of a publication, and carries out unique association at tPA and tPA MUTEIN The mouse hybridoma TPA 1-41 was obtained as a hybridoma which produces MoAb. The immunoglobulin class of an antibody TPA 1-41 and the subclass were IgG2bs (kappa chain) in measurement by the Ouchterlony technique.

[0039]

[The example 11 of reference] Isolation of the construction (1) cDNA content plasmid of the plasmid containing the gene which carries out the code of Homo sapiens tPA: Distribution were received for the cDNA library which made the host Escherichia coli x1776 which included cDNA compounded from the Homo sapiens prepuce origin primary culture cell mRNA in the pCD vector [Okayama et al., molecular cel biology (Molecular Cel1 Biology), and 3,280 (1983) reference], and created it from Dr. Okayama of the Osaka University microorganism disease lab. It is an alkaline process (plasmid DNA was extracted by Birnboim.H.C.& Doly.J., a NUKUREIKKU ASHIZZU research (Nucleic Acids Research), 1, and 1513 (1979)], this DNA was infected with Escherichia coli DH1, and the cDNA library which made the host Escherichia coli DH1 which consists of about 2x106 clone(s) was created.) from this cDNA library.

[0040] the cDNA library using above-mentioned Escherichia coli DH1 -- a nitrocellulose filter (Millipore Corp., HATF filter) top -- about -- ten sheets were wound so that it might become 5x104 clone / filter, and a total of 20 replica filters which made 1 set every two sheets each which are using this filter as the master filter was created. The Escherichia coli on this replica filter was melted with 0.5N NaOH solution, and the plasmid DNA which carried out exposure denaturation was fixed on the filter [Grunstein, M.& Hogness, D.S., and proceedings OBUNASHONARU academy OBU Science (Proc.Natl.Acad.Sci.USA) 72 and 3961 (1975)].

[0041] On the other hand It carries out based on the amino acid sequence of 301 and 214(1983)] Homo sapiens tPA. [Nature (Nature) reported by D.Pennica and others -- Amino acid No.248-260 (array number 3) (Asn-Arg-Arg-Leu-Thr-Trp-Glu-Tyr-Cys-Asp-Val-Pro-Ser), Amino acid No.489-501 (Arg-Met-Thr-Leu-Val-Gly-Ile-Ile-Ser-Trp-Gly-Leu-Gly) And amino acid No.516-527-stop (array number 4) () [Asn-Tyr-Leu-Asp-Trp-Ile-Arg-Asp-Asn-Met-A] rg-Pro-Stop (array number 5) On a basis the base sequence corresponding to these amino acid sequences oligonucleotide each which it has AAC CGC AGG CTG ACG TGG GAG TAC TGT GATGTG CCC TCC (Array number 6); CGC ATGACT TTG GTG GGC ATC ATC AGC TGG GGCCTG GGC (array number 7); Chemosynthesis of AAC TAC CTA GAC TGG ATT CGT GAC AAC ATG

CGA CCGTGA (array number 8) was carried out. 37 degrees C was made to react for 1 hour using T-four polynucleotide kinase (TAKARA SHUZO make) to this oligonucleotide in the reaction mixture [oligonucleotide 0.1microg, 50 mM Tris-HCl pH 8.0, 10mM MgCl₂, 10mM mercaptoethanol, 50microcuriegamma-32P ATP (>5000 Ci/mmole), and a 3 unit T-four polynucleotide kinase] of 50microl, and the indicator of the five prime end of an oligonucleotide was carried out by 32P. The replica filter which fixed DNA was made to meet separately by using as a probe two sorts of oligonucleotides which carried out the indicator by the above-mentioned approach. 5xSSPE[180mM NaCl, 10mM sodium phosphate buffer solution (pH7.4) with which a meeting reaction contains a 10microcurie probe, In 1mM EDTA], 5xDenhardt's, 0.1%SDS, and 10ml of 100microg [/ml] denaturation salmon sperm DNA solutions [T.Maniatis et al. who performed 35 degrees C for 16 hours, and washed the filter after a reaction 3 times every 30 with an every [30] of 45 more degrees C twice at the room temperature with the SDS solution 5xSSC [0.15M NaCl and 0.015M sodium-citrate buffer-solution] 0.1% -- Molecular cloning (Molecular Cloning) Cold Spring Harbor Laboratory, p.309(1982)].

[0042] radioautogram was taken from the washed filter and the strain which three kinds of probes boil all, and receives and reacts was looked for by piling up the radioautogram of the replica filter of two lots. It is 5x105 by this approach. One share [Escherichia coli K12 DH1/TPA13] which reacts from the colony of an individual to three kinds of probes was obtained. When extract purification of the plasmid DNA (pTPA13) was carried out with the alkaline process (a NUKUREIKKU ASHIZZU research (Nucleic Acids Research), and [1, 1513] (1979)) and the die length of cDNA was examined from this strain, it became clear that it is 2.3kb. However, in this cDNA chain length, it turned out that a code has not been carried out to an amino terminal side.

[0043] (2) *Homo sapiens* tPA Having made as *Escherichia coli* C600 the *Homo sapiens* neuroblastoma and cDNA library (Clonetec) which made the vector isolation lambda gt10 of the phage containing cDNA, by making Hf1A into a host, after winding ten sheets and moving this at a time on a nitrocellulose filter (Millipore Corp., HATF filter) about 1x105 clones on a soft-agar plate, desiccation immobilization of the phage DNA which melted and carried out exposure denaturation with 0.5N NaOH solutions was carried out on the filter. [Maniatis et al., "molecular cloning (Molecular Cloning)" Cold Spring Harbor Laboratory, p320, and 1982]. 32P indicator of the DNA fragment of 0.3Kb(s) which cut the plasmid pTPA13 obtained by (1) of the example 11 of reference on the other hand with restriction enzymes EcoRI and NarI, and are obtained was carried out with nick translation (Maniatis et al., same as the above p109), and it considered as the probe. An indicator

probe is included for the probe which carried out the indicator, and the filter which fixed DNA. 5xSSPE (the 0.9M NaCl 50mM sodium phosphate buffer solution (pH7.4) --) In 5mM EDTA, 50% formamide, 5xDenhardt's, 0.1% SDS, and 10ml of 100 microg [/ml] denaturation salmon sperm DNA solutions, 42 degrees C, the meeting reaction was performed, it washed at the room temperature in 2xSSC (1xSSC=0.15M NaCl, 0.015M sodium citrate) and a 0.1% SDS solution, and the filter after a reaction was washed every 30 twice at 68 degrees C in 1xSSC and 0.1%SDS solution every 30 twice for 16 hours. After drying the washed filter, radioautogram was taken and the probe and the clone which reacts were searched. The approach of the clone lambdaTPA7 twists Davis acquired by this approach [Davis et al., "advanced BAKUTE real genetics (Advanced Bacterial Genetics)", and Cold Spring Harbor Laboratory (1980)] As a result of extracting phage DNA and inquiring using several sorts of restriction enzymes, it turned out that clone lambdaTPA7 has cDNA which carries out the code of the amino terminal side of Homo sapiens tPA who is missing by pTPA13. Homo sapiens's tPA whole coding region was able to be covered by combining the cDNA parts of obtained pTPA13 and lambdaTPA7 the above result. Moreover, the base sequence of the obtained cDNA part was determined by the dideoxy NUKURECHIODO composition chain stopping method [Messing et al., "NUKUREIKKU ASHIZZU research (Nucleic Acids Research)" 9, and 309 (1981)]. As a result of comparing with the base sequence reported, the base sequence (No.552-554) CGG which carries out the code of amino acid No.129 (Arg) got down from TGG glue, and it turned out that the amino acid by which a code is carried out has placed and replaced Arg->Trp (array (drawing 3) number 9).

[0044] (3) lambdaTPA7 in which the plasmid for an animal cell manifestation carried out the construction above was cut by EcoRI, and the DNA fragment of 0.8kb was obtained. on the other hand -- plasmid pTB652[Science (Science) -- 236 : The fragment (about 4.2 kbs) including the ampicillin resistance gene which cut and obtained 1116-1120 (1987) and] by EcoRI, MuLV-LTR, and an SV40 promotor was prepared, both were ligation(ed), and the plasmid plambdaTPA seven E0.8 was built. The fragment containing tPA cDNA which digested and prepared the DNA fragment and plasmid pTPA13 of 2.1kbs which digested and obtained this plambdaTPA seven E0.8 by NarI and ClaI by NarI and ClaI was ligate(d), and the plasmid pTB920 was built (drawing 4). next, the DNA fragment of 2.7kbs which digested and obtained pTB920 by BamHI -- pTB399[cel structure and function (Cell Structure and Function) -- 12 : It inserted in the BamHI part of 208-217 and 1987], and pTB926 was built. This plasmid was digested by ClaI, the fragment of 4.0kbs was prepared, it included in the ClaI part of pTB348 (Cell Structure and Function 12:205-217, 1987), and the plasmid pTB927 was built (drawing 5).

[0045]

[The example 12 of reference] Homo sapiens -- tPA cDNA -- an animal cell -- a manifestation -- an ape -- COS - seven -- a cell -- [-- " -- a cel (Cell) -- " -- 27 -- 279 - 288 (1981) --] -- five -- % -- a new-born calf serum -- containing -- DMEM -- a culture medium -- monolayer culture (60mm plastics dish of diameters of a falcon) -- having carried out -- after -- said -- a culture medium -- culture-medium exchange -- having carried out. The calcium phosphate gel which contains DNA10microg of plasmids pTB920 or pTB927 per DISHU according to a well-known approach [Graham et al., "Virology (Virology)", 52, and 456 (1973)] 4 hours after exchange was prepared, it added into the cell, and pTB920 infected cell or pTB927 infected cell was obtained, respectively. Furthermore, culture of the pTB920 infection COS-7 above-mentioned cell or TB927 infection COS-7 cell was continued by the culture medium which carries out 4 hours after glycerol processing, and contains a new-born calf serum 5%. 70 - 72 hours after -- a culture supernatant -- collecting -- EIA given [the amount of tPA(s) in supernatant liquid] in the example 3 of reference -- when measured by law, 200microg-400microg [/ml] tPA was detected, and it was confirmed that Above cDNA is carrying out the code of Homo sapiens tPA correctly.

[0046]

[The example 13 of reference] The plasmid pTB927 of a publication was digested by Bgl II for the example 11 of manufacture reference of the recombinant DNA which has the base sequence which carries out the code of MUTEIN, the DNA fragment of 2.1kbs was obtained, this was further digested by ApaLI and BstYI, and the fragment of 1.3kbs was obtained. After graduating the both ends of this DNA by the reaction using klenow fragmentation, it included in the SmaI part of a plasmid pUC118, the plasmid pTB1127 was built, and it used as mold of the specific part directivity mutagenesis which uses an synthetic oligonucleotide. Specific part directivity variation was performed using Oligonucleotide-directed in vitro Mutagenesis System (Amersham) and synthetic oligomer. The array of the used synthetic oligomer is shown below.

5'-ACTGTTCCCTCAGACATATGAGGGGTGCTGCAGAA-3' (36mer) (array number 10)

By using this synthetic oligomer, while C173 was permuted by M, it was able to build Homo sapiens tPA cDNA into whom the recognition site of NdeI was introduced (drawing 6). That is, tPA MUTEIN which carried out deletion in FEK1 domain to the amino acid numbers 1-173 of tPA was obtained. Thus, the amino acid sequence guessed from tPA MUTEIN, the base sequence of tPA-1, and it which were obtained is shown in drawing 7 (array number 11). It is as follows if the difference from this MUTEIN and tPA is shown.

アミノ酸番号	174	295	303	527
t PAACSE.....	IFAKHRRSPGERFLP	
t PA-1MSE.....	IFAKHRRSPGERFLP	

The plasmid obtained as mentioned above was set to pTB1128 (drawing 8).
[0047]

[The example 14 of reference] pTB1128 obtained in the example 13 of the construction aforementioned reference of the plasmid pTB1133 for a MUTEIN manifestation of transformation (1) Homo sapiens tPA of Escherichia coli with the gene which carries out the code of Homo sapiens's tPA MUTEIN was cut by NdeI and BamHI, and the DNA fragment of about 1.1 kbs was obtained respectively. This fragment was inserted in the NdeI-BamHI part of plasmid pET3c [distribution were received from Studier and F.W. (Brookhaven National Labs.U.S.A.)], and the plasmid pTB1133 was built (drawing 8).

(2) Lambda phage DE3[Studier which included the RNA polymerase gene of T7 phage in a transformation, next 294 shares of Escherichia coli MM, F.W. et al., Journal OBU Molecular Biology (J.Mol.Biol.), 189 : Plasmid pLysS[Studier which is made to lysogenize 113-130 (1986)] and has the lysozyme gene of T7 phage further, and F.W. ** -- Journal OBU Molecular Biology (J.Mol.Biol.), 189 : 113-130 (1986)] was introduced and Escherichia coli MM294 (DE3) / pLysS stock was produced. pTB1133 was introduced into this Escherichia coli stock, and Escherichia coli MM294 (DE3) / pLysS, and pTB1133 were built. This strain was produced so that Homo sapiens tPA MUTEIN tPA-1 might be produced.

[0048]

[The example 15 of reference] Escherichia coli MM294/(DE3)pLysS obtained in the example 14 of culture reference of tPA-1 manifestation Escherichia coli, and PTB1133 were inoculated into 40ml of liquid media containing 1% bacto tryptan (Difco Laboratories, United States), 0.5% bacto yeast extractives (Difco Laboratories, United States), 0.5%NaCl, 100microg [/ml] ampicillin sodium, and 10microg [/ml] chloramphenicol in an Erlenmeyer flask with 200ml *****, and carried out shaking culture at 37 degrees C overnight. If 10ml of this culture medium was added to the 200ml of the above-mentioned liquid media containing 1% bacto tryptan, 0.5% bacto yeast extractives, 0.5%NaCl, 100microg [/ml] ampicillin sodium, and 10microg [/ml] chloramphenicol in 1,000ml *****, subsequently shaking culture of the isopropyl-beta-D-thio galactopyranoside (Wako Pure Chem Industries) 0.1mM was added and carried out at 37 degrees C for further 4 hours for 4 hours, and culture medium was obtained. Centrifugal separation of this culture medium was carried out, and fungus bodies were collected, and it

froze and saved at -80 degrees C.

[0049]

[The example 16 of reference] The tPA-1 manifestation Escherichia coli freezing fungus body obtained in the extract of tPA-1 protein, activation-izing and the purification ** extract, and the example 15 of activation-ized reference was suspended in 50ml (pH8.0) of 50mM tris hydrochloric-acid buffer solutions. After applying suspension to sonication (for [2] Ax2 minutes, 2 times) and obtaining lysate, at-long-intervals alignment separation was carried out by 18,900xg for 20 minutes, and settling were obtained. 50ml (pH8.0) of 10mM tris hydrochloric-acid buffer solutions washed these settling, at-long-intervals alignment separation was carried out by 18,900xg for 20 minutes, and settling were obtained. Furthermore 10mM tris hydrochloric-acid buffer solution (pH8.0) which contains Triton X-100 0.25% (v/v) washed these settling, at-long-intervals alignment separation was carried out by 18,900xg for 20 minutes, and settling were obtained. 25ml (7M guanidine hydrochloride-0.1M KH₂PO₄(pH7.5)-5mM 2-mercaptoethanol) of buffer solutions for an extract was added to these settling, and it agitated at 4 degrees C overnight. At-long-intervals alignment separation of this extract was carried out by 18,900xg for 20 minutes, and 25ml of extract supernatant liquid was obtained. 25ml of extract supernatant liquid -- 7M guanidine hydrochloride-0.1M Potassium phosphate buffer-solution (pH7.5)-5mM the object for activation-izing deaerated after dialyzing to 2-mercaptoethanol -- after agitating in addition to buffer-solution [2.5M urea-50mM tris hydrochloric-acid buffer-solution (pH8.75)-10mM NaCl-5mM EDTA-10mM lysine-0.5mM reduced glutathione-0.3mM oxidized glutathione] 1,250ml, in 15 degrees C, it was kept warm.

[0050] ** 1,250ml of each of tPA-1 solution formed into purification activation was dialyzed to -0.01% (pH8.0) of 50mM tris hydrochloric-acid buffer solutions Tween 80. At-long-intervals alignment separation of the liquid in dialysis was carried out by 18,900xg for 20 minutes, and dialysis supernatant liquid was obtained. Carry out a load to the QAE Toyo Perl 550C (TOSOH CORP.) column (70ml of capacity of column) which equilibrated this dialysis supernatant liquid with 50mM tris hydrochloric-acid buffer solution (pH8.0), and protein was made to adsorb, and after this buffer solution next washed the column, tPA-1 was eluted in the straight-line concentration gradient elution method (elution buffer-solution capacity of 600ml) of 0 - 1.0M NaCl. Carry out a load to the BENZAMI gin sepharose 6B (Pharmacia Corp., Sweden) column (20ml of capacity of column) which equilibrated the activity fraction by Tween80 -0.01% (pH8.0) of 0.5M NaCl-50mM tris hydrochloric-acid buffer solutions, and active tPA-1 was made to adsorb, and after this buffer solution next washed the column, active tPA-1 was

eluted with -0.01% (pH8.0) of 0.2M arginine-0.5M NaCl-50mM tris hydrochloric-acid buffer solutions Tween 80. By the above purification actuation, tPA-1 preparation which shows a single band by SDS-PAGE was obtained. That is, molecular weight was 35,000 under 39,500 and nonreduction conditions under reduction conditions.

[0051]

[The example 17 of reference] The plasmid pTB1128 of a publication was used for the example 13 of manufacture reference of the recombinant DNA which has the base sequence which carries out the code of MUTEIN (tPA-6') as mold of the specific part directivity mutagenesis which uses an synthetic oligonucleotide. Specific part directivity variation was performed using Oligonucleotide-directed in vitro Mutagenesis System (Amersham) and synthetic oligomer. The array of the used synthetic oligomer is as being shown below.

5'-GGGCGACTCTCGTGCTTGGCAAA-3' (24mer) (array number 12)
By using this synthetic oligomer, while R298'299 were permuted by E298'299, they were able to build FEK1 domain deletion Homo sapiens tPA cDNA into whom the recognition site of *Mbo*II was introduced (drawing 9). The amino acid sequence guessed from the base sequence of tPA MUTEIN and tPA-6' and it which were obtained using this synthetic oligomer is shown in drawing 10 (array number 13). It is as follows if the difference from this MUTEIN and tPA is shown.

アミノ酸番号	174	295	303	527
t P AACSE.....	IAKHEE	SPGERFL.....P	
t P A - 6'	-----MSE-----	IAKHEE	SPGERFL.....P	

The plasmid obtained as a result of using this synthetic oligomer was set to pTB1038.

[0052]

[The example 18 of reference] pTB1038 obtained in the example 17 of the construction aforementioned reference of the plasmid pTB1277 for a MUTEIN manifestation of transformation (1) Homo sapiens tPA of *Escherichia coli* with the gene which carries out the code of Homo sapiens's tPA MUTEIN (tPA-6') was cut by *Nde*I and *Bam*HI, and the DNA fragment of about 1.1 kbs was obtained. This fragment was inserted in the *Nde*I-*Bam*HI part of plasmid pET3c [distribution were received from Studier and F.W. (Brookhaven National Labs.U.S.A.)], and the plasmid pTB1277 was built (drawing 9).

(2) pTB1277 was introduced into *Escherichia coli* MM294 (DE3) / pLysS stock shown in the transformation, next the example 14 of reference, and *Escherichia coli* MM294 (DE3) / pLysS, and pTB1277 were built. Such strain

was produced so that *Homo sapiens* tPA MUTEIN tPA-6' might be produced.

[0053]

[The example 19 of reference] It cultivated under *Escherichia coli* MM294 (DE3) / *plysS* obtained in the example 18 of extract [of tPA-6' protein], activation-izing, and purification ** extract reference, and the culture condition which showed pTB1277 in the example 15 of reference, and the tPA-6' manifestation *Escherichia coli* object was acquired. 20g of this tPA-6' manifestation *Escherichia coli* fungus body was suspended in 0.2l. (pH8.0) of 50mM tris hydrochloric-acid buffer solutions. Suspension was applied to sonication (for [2] Ax2 minutes, 2 times), subsequently at-long-intervals alignment separation was carried out by 18,900xg for 20 minutes, and precipitate was obtained. 0.2l. (pH8.0) of 50mM tris hydrochloric-acid buffer solutions which contain Triton X-100 0.5% (v/v) washed this precipitate, at-long-intervals alignment separation was carried out by 18,900xg for 20 minutes, and precipitate was obtained. Furthermore 0.2l. (pH8.0) of 50mM tris hydrochloric-acid buffer solutions washed this precipitate, at-long-intervals alignment separation was carried out by 18,900xg for 20 minutes, and precipitate was obtained. 0.1l. (7M guanidine hydrochloride-0.1M KH₂PO₄(pH7.5)-0.1M 2-mercaptoethanol) of buffer solutions for an extract was added to this sediment, and it agitated at 4 degrees C overnight. At-long-intervals alignment separation of this extract was carried out by 18,900xg for 20 minutes, and 0.1l. of extract supernatant liquid was obtained. ** Buffer-solution [2.5M urea for the formation of activation-ized activation After adding 0.1l. of extract supernatant liquid to 50mM tris hydrochloric-acid buffer-solution (pH8.75)-10mM NaCl 5mM EDTA-10mM lysine 0.5mM reduced glutathione]20 l. and agitating at 15 degrees C for 1 hour, in addition, the oxidized glutathione was kept warm at 15 degrees C so that it might be set to last concentration 0.3mM. ** After condensing the tPA-6' solution formed into purification activation to 2l. with the ultrafiltration method and then dialyzing it to 50mM tris hydrochloric-acid buffer-solution (pH8.5) 0.01% Tween80, at-long-intervals alignment separation was carried out by 18,900xg for 20 minutes, and 2l. of dialysis supernatant liquid was obtained. Carry out a load to the QAE Toyo Perl 550C (TOSOH CORP.) column (150ml of capacity of column) which equilibrated this dialysis supernatant liquid with 50mM tris hydrochloric-acid buffer solution (pH8.5), and protein was made to adsorb, and after the 0.3l. of the same buffer solutions next washed the column, tPA-6' was eluted using the linear gradient elution method (elution buffer-solution capacity of 1,200ml) of 0 - 1.0M NaCl. 0.2l. of activity fractions was collected and it dialyzed to -0.01% (pH8.5) of 50mM tris hydrochloric-acid buffer solutions Tween 80. The load was carried out to the lysine sepharose 4B (Pharmacia

Corp., Sweden) column (40ml of capacity of column) which equilibrated 0.2l. of liquid in this dialysis with the same buffer solution, and tPA-6' was made to stick to it. -0.01% (pH8.5) of -0.01% (pH8.5) Tween80 0.2M arginine-0.5M NaCl-50mM tris hydrochloric-acid buffer solutions Tween 80 of 0.5M NaCl-50mM tris hydrochloric-acid buffer solutions after washing a column by 80 ml tPA-6' was eluted. By the above purification actuation, 38mg of tPA-6' preparations which show a single band by SDS-PAGE was obtained. That is, molecular weight was 39,000 under reduction conditions, and was 33,000 under nonreduction conditions. This value was well in agreement with the theoretical value (tPA-6'=39,561) presumed from the primary structure.

[0054]

[The example 20 of reference] The **-ed hybridoma culture supernatant was added on the anti-activated platelet sensitization plate produced in the example 2 for anti-activated platelet-tPA MUTEIN hybrid antibody measurement of EIA reference, and it was made to react to it at a room temperature for 2 hours. Subsequently, tPA which carried out the after [washing] biotin indicator by PBS-Tw was added, and it was made to react at a room temperature further for 2 hours. It measured by the approach which showed the HRP activity which added avidin-HRP complex next and was combined with solid phase after the 1-hour reaction at the room temperature to the example 1 of reference.

[0055]

[Example 1] preparation of the production ** immunogen of a mouse anti-Homo sapiens fibrin specific antibody production hybridoma, and tolerogen -- the peptide of A and B-2 kind which are shown in a degree type using a peptide synthesis machine (an applied system company, model 430A mold) with a well-known solid phase synthesis method was compounded.
A: H-Gly-His-Arg-Pro-Leu-Asp-Lys-Cys-OH (8-mer, array number 14)
B: H-Phe-Phe-Ser-Ala-Arg-Gly-His-Arg-Pro-Leu-Asp-Lys-OH (12-mer, array number 15)

Peptide A is a Homo sapiens fibrin beta chain amino terminal peptide (1-7). - It is equivalent to Cys and Peptide B is a fibrinogen beta chain partial peptide which consists of 12 amino acid which contains the above-mentioned fibrin beta chain amino terminal peptide (1-7) in C one end. Peptide A was added to the keyhole limpet hemocyanin (it may be hereafter written as KLH) beforehand maleimide-ized using meta-maleimide benzoyl-N-hydroxysuccinimide ester (it may be hereafter written as MBS), and the 7(peptide A)-KLH complex which peptide A 7 molecule combined per one mol of KLH(s) was prepared. After maleimide-izing Peptide B by MBS, by carrying out consecutive processing by S-acetyl mercapto and the succinic anhydride, and the hydroxylamine by one side, it added to D-GL [the copolymer:molecular weight 34 and 300 of D-glutamic acid and D-lysine and

D-Glu:D-Lys=60:40 (made in Pierce Chemicals)] which introduced the sulfhydryl group, and prepared the 4(peptide B)-D-GL complex which the peptide B4 molecule combined per D-GL1 molecule.

[0056] ** The immunization are using 7-KLH complex as immunization immunogen (peptide A), and using 4-D-GL complex as tolerogen (peptide B) was adopted. 1mg of tolerogens, 400micro [of physiological saline solutions] 1 / mouse was first injected intraperitoneally to the BALB/c mouse (**;n=10) five days and three days before the priming. subsequently, the Freund's incomplete adjuvant of equivalence [solution / of immunogen / physiological saline] -- adding -- enough -- after emulsion, and 40micro g/0.2ml / mouse -- the abdominal cavity -- and regions-of-back hypodermic administration was carried out. Although each of the 2-4th boosters emulsionized immunogen in the Freund's incomplete adjuvant and administered the first time and tales doses hypodermically, they injected intraperitoneally 500micro [of ***** tolerogens] g/200micro [of physiological saline solutions] 1 / mouse each five day and three days before the booster. About the individual which showed the blood serum antibody titer greatest in ten days after 4 times immunity, immunogen (50micro g/0.2ml / mouse) was administered intravenously.

** According to the approach of a publication, cell fusion was carried out to example of cell fusion reference 8-**.

** The hybridoma was screened in EIA of a publication for the examples 5 and 1 of reference using selection, cloning fibrinogen, and fibrin monomer joint plate of a hybridoma, and the anti-Homo sapiens fibrin specific antibody production hybridoma FTB 2-133 was hereafter acquired in the same way as example of reference 8-**. The obtained result was as having been shown in drawing 11 . In this drawing, the reactivity (O: EIA given in the example 5 of reference) over a fibrinogen and the reactivity (-: EIA given in the example 1 of reference) over a fibrin are shown.

[0057] The anti-Homo sapiens fibrin specific antibody production hybridoma FTB 2-133 showed the binding affinity only to the fibrin, and hardly reacted to the fibrinogen. The immunoglobulin class of two to FTB133 antibody and the subclass were IgG1 (kappa chain) in measurement by the Ouchterlony technique.

[0058] ** Two to FTB133 cell strain of the HAT resistance acquired by acquisition ** of a HAT sensitive strain was first cultivated by the 8-AZG addition culture medium of 1micro M concentration, subsequently 8-AZG concentration was raised every two to 5 times one by one, and culture was continued. HAT susceptibility and anti-Homo sapiens fibrin specific antibody production nature were measured about the cell strain which became resistance at 8-AZG of 100microM, and the target cell strain was selected. The obtained cell strain has antibody production nature equivalent to the

original HAT resistance stock, and became extinct 100% by the HAT addition culture medium.

[0059]

[Example 2] Manufacture of mouse anti-fibrin-anti-tPA MUTEIN bsMoAb (1)

** Immunity of the mouse was carried out in the same way as example of reference 8-** using the tPA-1 100 microg [/ml] physiological saline solution produced in the example 16 of immunity reference.

** The spleen was extracted in three days after the cell fusion last immunity, and spleen cell suspension was prepared with the conventional method (about 108 pieces). Subsequently, anti-Homo sapiens fibrin antibody production hybridoma FTB 2-133 of the HAT susceptibility acquired by example of reference 1-** 2x107 pieces were added and cell fusion and HAT selection were carried out in the way shown in example of reference 8-**.

** The hybrid hybridoma was screened using EIA of a publication for the example 6 of reference which measures EIA selection of a hybrid hybridoma, and given in the example 3 of reference using a cloning tPA joint microplate, and anti-fibrin-anti-tPA MUTEIN bsMoAb activity, and anti-fibrin-anti-tPA MUTEIN bsMoAb production TORIOMA TAF 1-42, TAF 1-79, and TAF 1-228 were acquired by the same approach as example of reference 8-** below.

The result of having presented EIA with each TORIOMA culture supernatant is shown in Table 1.

[0060] [Table 1] TORIOMA An absorbance (492nm) Anti-tPA antibody activity 1 Bispecific antibody activity 2 TAF 1-42 1.476 1.467 TAF 1-79 1.475 1.419 TAF 1-228 1.451 0.658 Control 3 0.051 Example of 0.1991 reference 3 reference.

2) Example of reference 6 reference.

3) Use the culture supernatant of an FIB 1-11 antibody-production hybridoma (refer to example of reference 8-**).

Three sorts of bsMoAb(s) All were IgG1 (kappa chain) as a result of measurement according [TAF 1-42, 1-79, and 1-228] to an Ouchterlony technique.

[0061] ** Intraperitoneal inoculation of bsMoAb production TORIOMA TAF 1-42 of 5x106 pieces / mouse, TAF 1-79, and TAF 1-228 was carried out at five BALB/c mouse 1 groups which injected intraperitoneally the ***** 0.5ml mineral oil of a hybrid antibody, respectively. Storage of ascites was seen about 10 - 15 days after. Purification of an antibody is a Homo sapiens fibrin beta chain amino terminal peptide (1-11) given [with a conventional method] in example of reference after fractionation 8-** with a 45-50% saturation ammonium sulfate. - The SERURO fine column which combined Cys was presented and the IgG fraction which has anti-fibrin antibody activity with affinity chromatography was acquired. Subsequently, the high performance chromatography using a hydroxyapatite column separated and

refined bsMoAb. In this way, bsMoAb TAF 1-42, TAF 1-79, and 12mg of 4mg of 30mg of TAF(s) 1-228 were obtained each from 5ml of ascites, respectively. The obtained result is shown in drawing 12. The peak shown by the arrow head was the IgG fraction which showed the bsMoAb activity positivity to the example 6 of reference in EIA of a publication.

[0062]

[Example 3] About the anti-tPA antibody production hybridoma TPA 1-41 acquired by the anti-Homo sapiens fibrin antibody production hybridoma FIB 1-11 acquired by example of manufacture (2) ** cell fusion reference 8-** of mouse anti-fibrin-anti-tPA MUTEIN bsMoAb, and example of reference 10-**, it is 0.5microg/ml, respectively. FITC and 1.5microg/ml By TRITC content Isco Huu Ham F and 12 mixing culture medium, it incubated for 30 minutes and fluorescent staining of the 37 degrees C was carried out. Subsequently, after adding the LSM solution (Wako Pure Chem industrial KK. sale) and removing a dead cell, both hybridomas were blended at a rate of 1:1 and cell fusion was carried out to example of reference 8-** by the approach of a publication using PEG6000. presenting FACS after 2-hour incubation at 37 degrees C -- a fluorescein and 25,000 cells by which the rhodamine double stain was carried out -- isolating preparatively -- a degree -- as a feeder -- mouse thymocyte -- 5x105 pieces / well -- to 96 hole microplate which carried out seeding, ten pieces / well came out comparatively, and seeding of the above-mentioned double-stain cell was carried out, and it was cultivated to it.

** EIA given in the examples 1, 3, and 6 of reference was presented with the culture supernatant of the well as which cell proliferation was regarded in one to two weeks after selection of a hybrid hybridoma, and cloning fusion, respectively, and antibody activity was measured. Cloning by limiting dilution was carried out about the well which showed high hybrid antibody activity, and the target bispecific antibody production mouse hybrid hybridoma (tetra-OMA) FT 2-14 was acquired.

[0063] ** Intraperitoneal inoculation of the mouse hybrid hybridoma FT 2-14 of 5x106 pieces / mouse was carried out at six BALB/c mice which injected intraperitoneally the ***** 0.5ml mineral oil of a hybrid antibody. Since storage of ascites was accepted about 10 - 20 days after, it was extracted, and it salted out with the 45-50% saturation ammonium sulfate, and the IgG fraction was obtained. Subsequently, 20mM(s) The fibrin joint SERURO fine column which equilibrated by PBS was presented, and it was eluted with 0.2M glycine and the hydrochloric-acid buffer solution (pH 2.9). It is 1N about the obtained protein fraction. The high performance chromatography using the hydroxyapatite column which dialyzed with 10mM potassium phosphate buffer solution (pH 6.8) after neutralization by NaOH, and equilibrated with the same buffer solution was presented. The duplex singularity hybrid

antibody FT 2-14 was refined using the salt concentration gradient elution method of potassium phosphate buffer-solution (pH 6.8) 10mM to 210mM(s). The 47mg purification antibody FT 2-14 was acquired from about 50ml antinode water. Obtained purification bsMoAb The result to which EIA for anti-fibrin-anti-tPA MUTEIN hybrid antibody measurement of a publication presented the example 6 of reference with FT 2-14 is shown in drawing 14. It became clear to have a Homo sapiens fibrin and avidity strong against Aikata of tPA-6'.

[0064] ** the purification profit **** purification antibody FT 2-14 of F(ab')2 was dissolved in 20mM acetic-acid buffer solution (pH 3.5), and the pepsin insolubilization column (5mg pepsin / 2.5ml SERURO fine gel) was presented. In 37 degrees C, it was slowly eluted in the 3ml [/hour] rate of flow, and the pepsin digest was obtained. 1N The protein fraction which presents after [adjustment.] protein A and a column by NaOH pH 7.5, and is eluted in PBS (pH 7.5) was isolated preparatively. Furthermore the fibrin joint column was presented and F(ab')2 fraction which has a column by PBS and has fibrin avidity with the glycine-hydrochloric-acid buffer solution of pH 2.5 after elution and washing was eluted. About 40mg FT 2-14 (intact-IgG molecule) to 4.3mg FT 2-14 F(ab')2 fraction was acquired.

** EIA given [F(ab')2 fraction obtained by bispecific antibody activity **] in the example 6 of reference was presented, and bispecific antibody activity was measured. The obtained result was as having been shown in drawing 14. An anti-[the inside of drawing and] fibrin, and anti-tPA MUTEIN bsMoAb The result related with FT 2-14 (-) and its F(ab')2 fraction (O) is shown. FT 2-14 F(ab')2 fraction showed the fibrin and the binding affinity strong against both of tPA MUTEIN like all IgG molecules.

[0065]

[Example 4] Mouse anti-fibrin-anti-tPA MUTEIN bsMoAb obtained by tPA-6' (the 0.5microg [/ml] last concentration) given in tPA (the 0.1microg [/ml] last concentration) of the preparation constant rate of immune complex liquid and the example 19 of reference in the example 2 of various concentration was added, the immunoreaction was carried out for 20 minutes at the room temperature, and immune complex liquid was prepared.

[0066]

[Example 5] the enhancing effect of fibrinolysis ability -- according to the well-known approach [D.Collen:SURON baud cis- - and - HEMOSUTASHISU (Thromb.Haemostasis) .45, and 225 (1981)], the plasma clot dissolution trial (plasma clot lysis assay) was carried out. That is, human plasma was added to the immune complex obtained in the example 4, and, subsequently the human thrombin was made to solidify in addition so that it may be set to last concentration 1.0 unit / ml. The turbidity of plasma was observed with time using the solubility analyzer (Euglobulin lysis analyzer "ELT-6" MebanixCo.),

and the time amount which the dissolution takes was measured. To the unqualified type tPA, three sorts of bsMoAb(s) [no] (TAF 1-42, TAF 1-79, and TAF 1-228) showed the enhancing effect of fibrinolysis ability. Although two sorts of bsMoAb(s) (TAF 1-42 and TAF 1-79) did not show an enhancing effect to tPA-6', as shown in drawing 13, as for TAF 1-228, the enhancing effect of a maximum of 5 times or more was shown to the dosage dependence target as fibrinolytic activity of tPA-6'. the time of an axis of ordinate making 100% dissolution ability of non-modified tPA single ** (-) or tPA MUTEIN tPA-6' single ** (O) among drawing -- respectively -- tPA / antibody complex (-), or tPA-6' -- the dissolution ability of /antibody complex (O) is expressed.

[0067]

[Example 6] the examples 9 and 10 of purification reference of the manufacture ** single singularity antibody of mouse anti-activated platelet-tPA MUTEIN and bsMoAb -- respectively -- a publication -- anti-- using activation platelet antibody production hybridoma 2T60 and the anti-tPA MUTEIN antibody production hybridoma TPA 1-41, antinode hydration was carried out according to the approach given in example 2-**, it refined further, and the IgG antibody fraction was obtained.

** two sorts of antibody IgG fractions prepared by the preparation above-mentioned ** of F(ab') 2 -- respectively well-known approach [-- M: -- Mariani:molecular immunology (Mol.Immunol.), 28, and 69(1991)] were presented, and it decomposed in tris and the hydrochloric-acid buffer solution (pH7.0) using ficin (sigma company sale). After adding L-cysteine (Wako Pure Chem sale) and activating a reaction (last concentration 1mM), it incubated at 37 degrees C for 4 hours. 100mM(s) The antibody fragment solution obtained after the reaction halt by addition of N-ethyl malei mide was dialyzed by PBS (pH 7.4). The protein A column (2.6x4.7cm) which equilibrated each antibody solution by the same PBS was presented, and the **** Li fraction was isolated preparatively. Furthermore, the gel filtration column chromatography using TSK gel (Oriental soda manufacture) was presented, and F(ab')2 fraction was prepared.

** Well-known approach [M. [2 / of the 2T 60 antibody origin prepared by preparation above-mentioned ** of hetero DAIME rucksack F(ab') 2 / F(ab')] after reduction and using o-phenylenedimaleimide in DTT J.Glennie:journal OBU immunology (J. Immunol.), 139, and 2367(1987)] are followed, and it is 2T60. The sulphydryl group of a Fab' fraction was maleimide-ized. F(ab') 2 of the TPA1-41 antibody origin prepared by the above-mentioned ** on the other hand -- the same -- DTT -- after reduction and a sephadex G25 gel-filtration column chromatography -- using -- superfluous DTT -- separation -- or -- removing -- the above-mentioned maleimide-izing -- 2T60 It added by the mole ratio 1:1 to Fab', and antibody Fab' which has two

sorts of different singularity by thioether association was combined with the hetero DAIME rucksack. The gel filtration column chromatography using URUTORO gel AcA44 column (LKB sale) was presented with the reaction mixture obtained, and purification preparative isolation of the hetero DAIME rucksack F(ab') 2 was carried out. The target duplex singularity F(ab') 2 was acquired for about 11mg of antibodies from 10mg of both ***** antibody purification IgG preparations.

** EIA given [hetero DAIME rucksack F(ab')2 fraction prepared by bispecific antibody activity **] in the example 20 of reference was presented, and anti-activated platelet-anti-tPA MUTEIN bispecific antibody activity was measured. The obtained result was as having been shown in drawing 15 .

[0068]

[Effect of the Invention] It combines with this tPA MUTEIN, without spoiling the thrombolysis ability of tPA MUTEIN, and the hybrid bsMoAb of this invention can be sharply increased in the compatibility to a thrombus, and selectivity. Therefore, by producing 1:1 immune complexes of Hybrid MoAb and tPA MUTEIN, it becomes the dissolution of an alternative and efficient thrombus, and removable.

[0069]

[0070]

[Layout Table]

array number: -- die-length [of one array]: -- mold [of 12 arrays]: -- amino acid topology: -- class [of straight chain-like array]: -- peptide fragmentation mold: -- amino terminal fragmentation

配列

Gly His Arg Pro Leu Asp Lys Lys Arg Glu Glu Cys

1 5 10.

[0071] array number: -- die-length [of two arrays]: -- mold [of 16 arrays]: -- amino acid topology: -- class [of straight chain-like array]: -- peptide fragmentation mold: -- pars intermedia fragmentation array Phe Phe Ser Ala Arg Gly His Arg Pro Leu Asp Lys Lys Arg Glu Glu 1 5 10 15.

[0072] array number: -- die-length [of three arrays]: -- mold [of 13 arrays]: -- amino acid topology: -- class [of straight chain-like array]: -- peptide fragmentation mold: -- pars intermedia fragmentation array Asn Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser 1 5 10.

[0073] array number: -- die-length [of four arrays]: -- mold [of 13 arrays]: -- amino acid topology: -- class [of straight chain-like array]: -- peptide fragmentation mold: -- pars intermedia fragmentation array

Arg-Met-Thr-Leu-Val-Gly-Ile-Ile-Ser-Trp-Gly-Leu-Gly 1 5 10.

[0074] array number: -- die-length [of five arrays]: -- mold [of 12 arrays]:

-- amino acid topology: -- class [of straight-line-like array]: -- peptide fragmentation mold: -- C terminal fragmentation-stop
配列

Asn-Tyr-Leu-Asp-Trp-Ile-Arg-Asp-Asn-Met-Arg-Pro

1 5 10.

[0075] array number: -- die-length [of six arrays]: -- mold [of 39 arrays]:
-- nucleic-acid-topology: -- nucleic acid besides class: of a straight chain-like array Synthetic DNA array AAC CGC AGG CTG ACG TGG GAG TAC TGT GAT GTG CCC TCC 39.

[0076] array number: -- die-length [of seven arrays]: -- mold [of 39 arrays]: -- nucleic-acid-topology: -- nucleic acid besides class: of a straight chain-like array Synthetic DNA array CGC ATG ACT TTG GTG GGC ATC ATC AGC TGG GGC CTG GGC 39.

[0077] array number: -- die-length [of eight arrays]: -- mold [of 39 arrays]: -- nucleic-acid-topology: -- nucleic acid besides class: of a straight chain-like array Synthetic DNA array AAC TAC CTA GAC TGG ATT CGT GAC AAC ATG CGA CCG TGA 39.

[0078] array number: -- die-length [of nine arrays]: -- mold [of 1800 arrays]: -- number [of nucleic-acid chains]: -- double strand topology: -- class [of straight chain-like array]: -- description existence location [of a cDNA to mRNA origin Homo sapiens prepuce origin primary culture cell array]: -- approach: which determined 552..554 description -- E array

AAAACCTCTG CGAGGAAAGG GAAGGAGCAA GCCGTGAATT
TAAGGGACGC TGTGAAGCAA 60TC ATG GAT GCA ATG AAG AGA GGG
CTC TGC TGT GTG CTG CTG CTG TGT 107 Met Asp Ala Met Lys Arg Gly
Leu Cys Cys Val Leu Leu Cys -35 - 30 - 25GGA GCA GTC TTC GTT
TCG CCC AGC CAG GAA ATC CAT GCC CGA TTC AGA 155 Gly Ala Val
Phe Val Ser Pro Ser Gln Glu Ile His Ala Arg Phe Arg -20 - 15 - 10 - 5AGA
GGA GCC AGA TCT TAC CAA GTG ATC TGC AGA GAT GAA AAA ACG
CAG 203 ArgGly Ala Arg Ser Tyr Gln Val Ile Cys Arg Asp Glu Lys Thr Gln 1
5 10 ATG ATA TAC CAG CAACATCAG TCA TGG CTG CGC CCT GTG
CTC AGA AGC 251 Met Ile Tyr GlnGln His Gln Ser Trp Leu Arg Pro Val Leu
Arg Ser 15 20 25AAC CGG GTG GAA TAT TGC TGG TGC AAC AGT GGC
AGG GCA CAG TGC CAC 299 Asn Arg Val Glu Tyr Cys Trp Cys Asn Ser
Gly Arg Ala Gln Cys His 30 35 40 TCA GTG CCT GTC AAAAGT TGC
AGCGAG CCA AGG TGT TTC AAC GGG GGC 347 Ser Val Pro Val Lys
SerCys Ser Glu Pro Arg Cys Phe Asn Gly Gly 45 50 55 60ACC TGC CAG
CAGGCC CTG TAC TTC TCA GAT TTC GTG TGC CAG TGC CCC 395 Thr
Cys Gln Gln Ala Leu Tyr Phe Ser Asp Phe Val Cys Gln Cys Pro 65 70 75
GAA GGA TTT GCT GGGAAAG TGC TGT GAA ATA GAT ACC AGG GCC

ACG TGC 443 Glu Gly Phe Ala Gly Lys Cys Cys Glu Ile Asp Thr Arg Ala Thr Cys 80 85 90TAC GAG GAC CAG GGC ATCAGC TAC AGG GGC ACG TGG AGC ACA GCG GAG 491 Tyr Glu Asp Gln Gly Ile Ser Tyr Arg Gly Thr Trp Ser Thr Ala Glu 95 100 105AGT GGC GCC GAG TGC ACC AACTGG AAC AGC AGC GCG TTG GCC CAG AAG 539 Ser Gly Ala Glu CysThr Asn Trp Asn Ser Ser Ala Leu Ala Gln Lys 110 115 120CCC TAC AGT GGG TGG AGG CCA GACGCC ATC AGG CTG GGC CTG GGG AAC 587 Pro Tyr Ser Gly Trp ArgPro Asp Ala Ile Arg Leu Gly Leu Gly Asn 125 130 135 140CAC AACTAC TGC AGA AAC CCA GAT CGA GAC TCA AAG CCC TGG TGC TAC 635 His AsnTyr Cys Arg Asn Pro Asp Arg Asp Ser Lys Pro Trp Cys Tyr 145 150 155 GTC TTT AAG GCG GGG AAG TAC AGC TCA GAG TTC TGC AGC ACC CCT GCC 683 Val Phe Lys Ala Gly Lys Tyr Ser Ser Glu Phe Cys-Ser-Thr-Pro-Ala 160 165 170TGC TCT GAG GGA AAC AGT-GAC-TGC-TAC-TTT GGG AAT GGG TCA GCC TAC 731Cys Ser Glu-Gly-Asn-Ser-Asp Cys Tyr Phe Gly Asn-Gly-Ser-Ala-Tyr 175 180 185CGT GGC ACG CAC AGC CTC ACC GAGTCG GGT GCC TCC TGC CTC CCG TGG 779 Arg Gly Thr His Ser LeuThr Glu Ser Gly Ala Ser Cys Leu Pro Trp 190 195 200AAT TCC ATG ATC CTG ATA GGC AAG GTT TAC ACA GCA CAG AAC CCC AGT 827 Asn Ser Met Ile Leu IleGly Lys Val Tyr Thr Ala Gln Asn Pro Ser205 210 215 220GCC CAG GCA CTGGGC CTG GGC AAA CAT AAT TAC TGC CGG AAT CCT GAT 875 Ala GlnAla Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp 225 230 235GGG GAT GCC AAG CCC TGG TGC CAC GTG CTG AAG AAC CGC AGG CTG ACG 923 Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr 240 245 250TGGGAG TAC TGT GAT GTGCC TCC TGC TCC ACC TGC GGC CTG AGA CAG 971 Trp Glu Tyr CysAsp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln 255 260 265TAC AGC CAG CCT CAG TTT CGC ATC AAA GGA GGG CTC TTC GCC GAC ATC 1019 Tyr Ser Gln Pro Gln PheArg Ile Lys Gly Gly Leu Phe Ala Asp Ile 270 275 280GCC TCC CAC CCC TGG CAG GCT GCC ATC TTT GCC AAGCACAGG AGG TCG 1067 Ala Ser His Pro Trp GlnAla Ala Ile Phe Ala Lys His Arg Arg Ser 285 290 295 300CCC GGA GAG CGG TTC CTG TGC GGG GGC ATA CTC ATC AGC TCC TGC TGG 1115 Pro GlyGlu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp 305 310 315 ATT CTC TCT GCC GCCCAC TGC TTC CAG GAG AGG TTT CCG CCC CAC CAC 1163 Ile Leu SerAla Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His 320 325 330CTG ACG GTG ATC TTG GGC AGA ACA TAC CGG GTG GTC CCT GGC GAG GAG 1211 Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu 335 340 345GAG CAG AAATT GAA GTC GAAAAA TAC ATT GTC CAT AAG GAA TTC GAT 1259 Glu Gln Lys Phe GluVal Glu Lys Tyr Ile Val His Lys Glu Phe Asp 350 355 360 GAT GAC ACT TAC GAC AAT GAC ATT GCG CTG CTG CAG CTG AAA TCG GAT 1307Asp A sp.Thr Tyr Asp Asn Asp-Ile-Ala-Leu-Leu Gln Leu Lys Ser Asp365 370 375 380TCG

TCC CGC TGT GCC CAG GAG AGC AGC GTG GTC CGC ACT GTG TGC
 CTT 1355Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg-Thr-Val-CysLeu
 385 390 395 CCC CCG GAG GAC CTGCAG CTG CCG GAC TGG ACG GAG
 TGT GAG CTC TCC 1403 Pro Pro GluAsp Leu Gln Leu Pro Asp Trp Thr Glu
 Cys Glu Leu Ser 400 405 410GGC TAC GGC AAG CAT GAG GCC TTG TCT
 CCT TTC TAT TCG GAG CGG CTG 1451 Gly Tyr Gly Lys His Glu Ala Leu
 Ser Pro Phe Tyr Ser Glu Arg Leu 415 420 425AAG GAG GCTCAT GTC AGA
 CTGTAC CCA TCC AGC CGC TGC ACA TCA CAA 1499 Lys Glu Ala His
 ValArg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln 430 435 440CAT TTA CTT
 AAC AGA ACA GTC ACC GAC AAC ATG CTG TGT GCT GGA GAC 1547
 His Leu Leu Asn Arg ThrVal Thr Asp Asn Met Leu Cys Ala Gly Asp445 450
 455 460ACT CGG AGC GGCGGG CCC CAG GCA AAC TTG CAC GAC
 GCC TGC CAG GGC 1595 Thr ArgSer Gly Gly Pro Gln Ala Asn Leu His Asp
 Ala Cys Gln Gly 465 470 475GAT TCG GGA GGC CCC CTG GTG TGT CTG
 AAC GAT GGC CGC ATG ACT TTG 1643 Asp Ser Gly Gly Pro Leu Val Cys
 Leu Asn Asp Gly Arg Met Thr Leu 480 485 490GTGGGC ATC ATC AGC
 TGGGGC CTG GGC TGT GGA CAG AAG GAT GTC CCG 1691 Val Gly Ile
 IleSer Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro 495 500 505GGT GTG
 TAC ACC AAG GTT ACC AAC TAC CTA GAC TGG ATT CGT GAC AAC
 1739 Gly Val Tyr Thr LysVal Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn 510
 515 520 ATG CGA CCG TGACCAAGGAA CACCCGACTC CTCAAAAGCA
 AATGAGATCC 1788Met Arg Pro525 527CGCCTCTTCT TC 1800.

[0079] array number: -- die-length [of ten arrays]: -- mold [of 36 arrays]:
 -- nucleic-acid-topology: -- nucleic acid besides class: of a straight
 chain-like array Synthetic DNA array ACTGTTCCC TCAGACATAT
 GAGGGGTGCT GCAGAA 36.

[0080] array number: -- die-length [of 11 arrays]: -- mold [of 1068 arrays]:
 -- number [of nucleic-acid chains]: -- double strand topology: -- nucleic
 acid besides class: of a straight chain-like array description existence
 location [of a qualification array of the array number 7]: -- approach which
 determined the amino acid deficit description to 1-173 :P Array ATG-TCT
 GAG GGA AAC AGT GAC TGC TAC TTT GGG AAT GGG TCA GCC TAC
 48 Met Ser Glu Gly Asn Ser Asp Cys Tyr Phe Gly Asn Gly Ser Ala Tyr1 5 10
 15CGT GGC ACG CAC AGC CTC ACC GAG TCG GGT GCC TCC TGC
 CTC CCG TGG 96 Arg Gly Thr His Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu
 Pro Trp 20 25 30AAT TCC ATG ATC CTG ATA GGC AAG GTT TAC ACA
 GCA CAG AAC CCC AGT 144 Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr
 Ala Gln Asn Pro Ser 35 40 45GCC CAG GCACTG GGC CTG GGCAA CAT
 AAT TAC TGC CGG AAT CCT GAT 192 Ala Gln Ala Leu GlyLeu Gly Lys His
 Asn Tyr Cys Arg Asn Pro Asp 50 55 60 GGG GAT GCC AAG CCC TGG
 TGC CAC GTG CTG AAG AAC CGC AGG CTG ACG 240 Gly Asp Ala Lys
 Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr65 70 75 80TGGGAG TAC

TGT GAT GTG CCC TCC TGC TCC ACC TGC GGC CTG AGA CAG288 Trp
Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln 85 90 95TAC
AGC CAG CCT CAG TTT CGC ATC AAA GGA GGG CTC TTC GCC GAC
ATC 336 Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile
100 105 110 GCC TCC CAC CCC TGGCAG GCT GCC ATC TTT GCC AAG
CAC AGG AGG TCG 384 Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His
Arg Arg Ser 115 120 125 CCC GGA GAG CGG TTC CTG TGC GGG GGC
ATA CTC ATC AGC TCC TGC TGG 432 Pro Gly GluArg Phe Leu Cys Gly
Gly Ile Leu Ile Ser Ser Cys Trp 130 135 140 ATT CTC TCT GCC GCC CAC
TGC TTC CAG GAG AGG TTT CCG CCC CAC CAC 480 Ile Leu Ser Ala Ala
His Cys Phe Gln Glu Arg Phe Pro Pro His His145 150 155 160CTG ACG
GTG ATC TTG GGC AGA ACA TAC CGG GTG GTC CCT GGC GAG GAG
528 Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu 165 170
175 GAG CAG AAA TTTGAAGTC GAA AAA TAC ATT GTC CAT AAG GAA
TTC GAT 576 Glu Gln LysPhe Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe
Asp 180 185 190GAT GAC ACT TAC GAC AAT GAC ATT GCG CTG CTG
CAG CTG AAA TCG GAT 624 Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu
Gln Leu Lys Ser Asp 195 200 205 TCG-TCC-CGC-TGT-GCC
CAG-GAG-AGC-AGC-GTG GTC CGC ACT GTG TGC CTT 672Ser Ser
Arg-Cys-Ala-Gln-Glu Ser Ser Val Val Arg Thr Val Cys Leu 210 215 220
CCC-CCG-GAG-GAC-CTG CAG CTGCCG GAC TGG ACG GAG TGT GAG
CTC TCC 720 Pro Pro Glu Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu
Leu Ser225 230 235 240GGC TAC GGC AAG CAT GAG GCC TTG TCT
CCT TTC TAT TCG GAGCGG CTG 768 Gly TyrGly Lys His Glu Ala Leu Ser
Pro Phe Tyr Ser Glu Arg Leu 245 250 255AAG GAG GCT CAT GTC AGA
CTG TAC CCA TCC AGC CGC TGC ACA TCA CAA 816 Lys Glu AlaHis Val
Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln 260 265 270CAT TTA CTT
AAC AGA ACAGTC ACC GAC AAC ATG CTG TGT GCT GGA GAC 864 His
Leu Leu AsnArg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp 275 280 285
ACT CGG AGC GGC GGGCCC CAG GCA AAC TTG CAC GAC GCC TGC
CAG GGC 912 Thr Arg SerGly Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln
Gly 290 295 300 GAT TCG GGAGGC CCC CTG GTG TGT CTG AAC GAT
GGC CGC ATG ACT TTG 960 Asp Ser Gly GlyPro Leu Val Cys Leu Asn Asp
Gly Arg Met Thr Leu305 310 315 320GTG GGC ATC ATC AGC TGG GGC
CTG GGC TGT GGA CAG AAG GAT GTC CCG 1008 Val Gly Ile Ile Ser Trp
Gly Leu Gly Cys Gly Gln Lys Asp Val Pro 325 330 335 GGT GTG TAC
ACCAAGGTT ACC AAC TAC CTA GAC TGG ATT CGT GAC AAC 1056 Gly
Val TyrThr Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn 340 345
350ATG CGA CCG TGA 1068Met Arg Pro 355.

[0081] array number: -- die-length [of 12 arrays]: -- mold [of 24 arrays]:
-- nucleic-acid-topology: -- nucleic acid besides class: of a straight
chain-like array Synthetic DNA array GGGCGACTCT TCGTGCTTGG CAAA

24.

[0082] array number: -- die-length [of 13 arrays]: -- mold [of 1068 arrays]: -- number [of nucleic-acid chains]: -- double strand topology: -- nucleic acid besides class: of a straight chain-like array The amino acid deficit to 1-173 description existence location [of a qualification array of the array number 9]: -- 298 and the 299 place amino-acid-substitution description Determined approach :P Array ATG TCT GAG GGA AAC AGT GAC TGC TAC TTT GGG AAT GGG TCA GCC TAC 48 Met Ser Glu Gly Asn Ser Asp Cys Tyr Phe Gly Asn Gly Ser Ala Tyr1 5 10 15CGT GGC ACG CAC AGC CTC ACC GAG TCG GGT GCC TCC TGC CTC CCG TGG 96 Arg Gly Thr HisSer Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp 20 25 30AAT TCC ATG ATC CTG ATAGGC AAG GTT TAC ACA GCA CAG AAC CCC AGT 144 Asn Ser Met IleLeu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser 35 40 45GCC CAG GCA CTG GGC CTG GGC AAA CAT AAT TAC TGC CGG AAT CCT GAT 192 Ala Gln Ala Leu GlyLeu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp 50 55 60 GGGGAT GCC AAGCCC TGG TGC CAC GTG CTG AAG AAC CGC AGG CTG ACG 240 Gly AspAla Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr65 70 75 80TGGGAG TAC TGT GAT GTG CCC TCC TGC TCC ACC TGC GGC CTG AGA CAG288 Trp Glu Tyr Cys AspVal Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln 85 90 95TAC AGCCAG CCT CAG TTT CGCATC AAA GGA GGG CTC TTC GCC GAC ATC336 Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile 100 105 110GCC TCCCAC CCC TGG CAG GCTGCC ATC TTT GCC AAG CAC GAA GAG TCG 384 Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Glu Glu Ser 115 120 125 CCC GGA GAG CGGTTCCCTG TGC GGG GGC ATA CTC ATC AGC TCC TGC TGG 432 Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp 130 135 140 ATT CTC TCT GCC GCC CAC TGC TTC CAG GAG AGG TTT CCG CCC CAC CAC 480 Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His145 150 155 160CTGACG GTG ATC TTG GGC AGA ACA TAC CGG GTG GTC CCT GGCGAG GAG 528 Leu ThrVal Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu 165 170 175 GAG-CAG-AAA-TTT-GAA GTC-GAA-AAA-TAC-ATT GTC CAT AAG GAA TTC GAT 576Glu Gln Lys-Phe-Glu-Val-Glu Lys Tyr Ile Val His-Lys-Glu-Phe-Asp 180 185 190GAT GAC ACT TAC GAC AAT GAC ATT GCG CTG CTG CAG CTG AAA TCG-GAT 624 Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp 195 200 205 TCG TCC CGC TGT GCC CAG GAG AGC AGC GTG GTC CGC ACT GTG TGC CTT 672 Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu 210 215 220 CCC CCG GAGGAC CTG CAG CTG CCG GAC TGG ACG GAG TGT GAG CTC TCC 720 Pro Pro Glu Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser225 230 235 240GGC TAC GGC AAG CAT GAG GCC TTG TCT CCT TTC TAT TCG GAGCGG CTG 768 Gly Tyr Gly Lys His Glu Ala Leu Ser Pro

Phe Tyr Ser Glu Arg Leu 245 250 255 AAG GAG GCT CAT GTCAGA CTG
TAC CCA TCC AGC CGC TGC ACA TCA CAA 816 Lys Glu AlaHis Val Arg
Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln 260 265 270CAT TTA CTT AAC
AGA ACA GTC ACC GAC AAC ATG CTG TGT GCT GGA GAC 864 His Leu
Leu Asn Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp 275 280 285
ACT CGG AGC GGC GGG CCC CAG GCA AAC TTG CAC GAC GCC TGC
CAG GGC 912 Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp Ala Cys
Gln Gly 290 295 300 GAT TCG GGAGGC CCC CTG GTG TGT CTG AAC
GAT GGC CGC ATG ACT TTG 960 Asp Ser Gly Gly Pro Leu Val Cys Leu
Asn Asp Gly Arg Met Thr Leu 305 310 315 320 GTG GGC ATC ATC AGC
TGG GGC CTG GGC TGT GGA CAG AAG GATGTC CCG 1008 Val Gly Ile
Ile Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro 325 330 335 GGT GTG
TAC ACC AAGGTT ACC AAC TAC CTA GAC TGG ATT CGT GAC AAC
1056 Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn 340
345 350 ATG CGA CCG TGA 1068 Met Arg Pro 355.

[0083] array number: -- die-length [of 14 arrays]: -- mold [of eight arrays]:
]: -- amino acid topology: -- class [of straight chain-like array]: -- peptide
fragmentation mold: -- amino terminal fragmentation
配列

Gly His Arg Pro Leu Asp Lys Cys

5.

[0084] array number: -- die-length [of 15 arrays]: -- mold [of 12 arrays]:
-- amino acid topology: -- class [of straight chain-like array]: -- peptide
fragmentation mold: -- pars intermedia fragmentation
配列

Phe Phe Ser Ala Arg Gly His Arg Pro Leu Asp Lys

5

10.

[Translation done.]

*** NOTICES ***

JPO and NCIPI are not responsible for any damages caused by the use of this translation.

1. This document has been translated by computer. So the translation may not reflect the original precisely.
- 2.**** shows the word which can not be translated.
3. In the drawings, any words are not translated.

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1] It is the mimetic diagram showing the structure of tPA and tPA MUTEIN.

[Drawing 2] It is the mimetic diagram showing the structure of IgG.

[Drawing 3] It is the amino acid sequence presumed from Homo sapiens's tPA cDNA base sequence and it which were obtained in the example 1 of reference.

[Drawing 4] It is the construction Fig. of a plasmid pTB920.

[Drawing 5] It is the construction Fig. of a plasmid pTB927.

[Drawing 6] It is the mimetic diagram of a specific part variation which was performed in obtaining Homo sapiens tPA MUTEIN tPA-1.

[Drawing 7] It is the amino acid sequence presumed from the base sequence of Homo sapiens tPA MUTEIN tPA-1, and it.

[Drawing 8] It is the construction Fig. of a plasmid 1128 and pTB 1133.

[Drawing 9] It is the construction Fig. of a plasmid 1038 and pTB 1277.

[Drawing 10] It is the amino acid sequence presumed from the base sequence of Homo sapiens tPA MUTEIN tPA-6', and it.

[Drawing 11] The antibody dilution curve of the anti-Homo sapiens fibrin specific antibody FTB 2-133 obtained in the example 1 is expressed. The reactivity (O: EIA given in the example 5 of reference) over a fibrinogen and the reactivity (-: EIA given in the example 1 of reference) over a fibrin are shown.

[Drawing 12] The purification result of the anti-fibrin and anti-tPA MUTEIN bsMoAb of a publication (TAF 1-42, TAF 1-79, and TAF 1-228) is expressed to example 2-(4). That is, an IgG fraction is acquired from the antinode water containing bsMoAb by salting-out processing, and after a fibrin beta chain amino terminal peptide linkage column refines further, the result with which the hydroxyapatite column was presented is shown. The 280nm absorbance of an eluate shows a chromatography pattern, and an arrow head shows the

peak of a bsMoAb activity positivity to the example 6 of reference in EIA of a publication.

[Drawing 13] An anti-fibrin and anti-tPA MUTEIN bsMoAb given in example 2-** It is in about TAF 1-228. The result when presenting a vitro plasma clot dissolution trial is expressed. the time of an axis of ordinate making 100% dissolution ability of non-modified tPA single ** (-) or tPA MUTEIN tPA-6' single ** (O) -- respectively — tPA / antibody complex (-), or tPA-6' -- the dissolution ability of / antibody complex (O) is expressed (example 5 reference).

[Drawing 14] An anti-fibrin and anti-tPA MUTEIN bsMoAb given in an example 3 The result of having measured FT 2-14 (-) and its F(ab')2 fraction (O) for the example 6 of reference in EIA of a publication is expressed.

[Drawing 15] The result of having measured the activity of the anti-activated platelet-anti-tPA MUTEIN bispecific antibody F(ab') 2 of a publication for the example 20 of reference in EIA of a publication is expressed to example 6-** (refer to example 6-**).

[Translation done.]

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(71)出願人 000002934

武田薬品工業株式会社

大阪府大阪市中央区道修町四丁目1番1号

(72)発明者 岩佐 進

京都府綴喜郡田辺町大住ヶ丘1丁目21番地
の2

(72)発明者 黒川 智文

兵庫県川西市大和西2丁目16番地の7

(72)発明者 豊田 幸生

兵庫県尼崎市水堂町1丁目8番8号

(74)代理人 弁理士 大多和 明敏 (外1名)

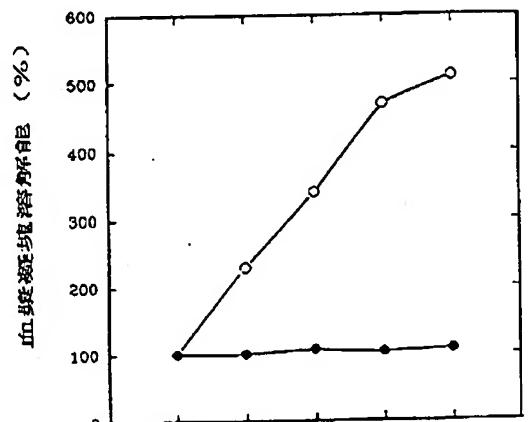
(54)【発明の名称】ハイブリッド・モノクローナル抗体および抗体含有薬剤

(57)【要約】

【目的】血栓溶解剤としての組織プラスミノーゲンアクトベーター(tPA)の血中半減期を延長及びtPAムテインの血栓への選択性の低下を改善し、またこれら血栓溶解剤の副作用を抑える。

【構成】二重特異性の一方が血栓に対し、他方がF、EおよびK1ドメインを欠失してなるtPA、例えばtPA-A-6'に対する二重特異性モノクローナル抗体、および該二重特異性モノクローナル抗体に、F、EおよびK1ドメインを欠失してなるtPAムテインを免疫結合させてなる血栓溶解剤。

【効果】血栓溶解能、血栓への選択性が増し、一方、フィブリノーゲンに対する反応性も少ないのでフィブリノーゲン分解能が高まるといった副作用もない血栓溶解剤が得られた。



二重特異性抗体/tPAもしくはtPA-A-6' (モル比)

1

【特許請求の範囲】

【請求項1】二重特異性の一方が血栓に対するものであり、他方がフィンガー(F)ドメイン、成長因子(E)ドメインおよびクリングル(K)1ドメインを欠失してなる組織プラスミノーゲンアクチベーター(tPA)ムテインに対するものである二重特異性抗体。

【請求項2】血栓がフィブリンである請求項1記載の二重特異性抗体。

【請求項3】血栓が活性化血小板である請求項1記載の二重特異性抗体。

【請求項4】少なくとも可変領域を含有し、かつ重鎖定常領域ドメイン2および3を欠失する請求項1記載の二重特異性抗体。

【請求項5】二重特異性抗体がF(a b')_nである請求項4記載の二重特異性抗体。

【請求項6】請求項1記載の二重特異性抗体に、F、EおよびK1ドメインを欠失してなるtPAムテインを、免疫結合させてなる血栓溶解剤。

【請求項7】tPAムテインがアミノ酸残基番号296から302の領域の一部ないし全部を欠失してなるものである、請求項6記載の血栓溶解剤。

【請求項8】tPAムテインがアミノ酸残基番号296から304の間のアミノ酸残基の一部ないし全部を別のアミノ酸残基に置換してなるものである、請求項6記載の血栓溶解剤。

【請求項9】tPAムテインがアミノ酸残基番号296から302の領域の一部ないし全部を欠失させ、かつアミノ酸残基番号296から304の領域の一部のアミノ酸残基を別のアミノ酸残基に置換してなるものである、請求項7記載の血栓溶解剤。

【発明の詳細な説明】

【0001】

【産業上の利用分野】本発明は、二重特異性を有するハイブリッド・モノクローナル抗体（以下、MoAbと略記することがある）に関する。さらに詳しくは、二重特異性の一方が血栓に対するものであり、他方がF、EおよびK1ドメインを欠失してなるtPAムテインに対するものである二重特異性モノクローナル抗体（以下、bsMoAbと略記することがある）に関する。本発明はまた、上記のbsMoAbに、F、EおよびK1ドメインを欠失してなるtPAムテインを免疫結合させてなる血栓溶解剤に関する。

【0002】

【従来の技術】心筋梗塞、動脈塞栓症あるいは脳梗塞などの血栓性患者には広く血栓溶解療法が用いられ、ストレプトキナーゼ（以下、SKと略記することがある）やウロキナーゼ（以下、UKと略記することがある）などが臨床応用されている。最近では、血栓への選択性が高く、従って出血傾向の副作用を軽減すると云われているティッシュ・プラスミノーゲン・アクチベータ（以下、

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tPAと略記することがある）やプロウロキナーゼ（以下、ProutUKと略記することがある）が登場し、上記のSKやUKに代わろうとしている。さらに、より効率の高い、かつ血栓選択性に優れた血栓溶解剤として修飾型tPAなども開発されてきた。また一方で、抗体ターゲティングを利用した血栓溶解剤も登場し[C. Bodeら：サイエンス(Science), 229, 765 (1985); M. S. Rungeら：プロシーディングス・オブ・ナショナル・アカデミー・サイエンス ユーエスエー(Proc. Natl. Acad. Sci. USA), 84, 7659 (1987); T. Kurokawaら：バイオテクノロジー(Bio/Technology), 7, 1163 (1989); ヨーロッパ特許出願第0363712公開公報参照]、血栓を形成するフィブリンや活性化血小板に高い親和性を有する抗体を利用して、血栓溶解作用物質単独に比べて数倍から数十倍効率の高い血栓溶解剤も作製されるようになった。特に血栓と血栓溶解作用物質との両者に免疫結合できるbsMoAbは、抗体活性および血栓溶解活性の低下を伴わない、きわめて効率的な血栓溶解剤を提供している。

【0003】

【発明が解決しようとする課題】しかしながら、新しい世代の血栓溶解剤として登場したtPAなどについても、半減期が非常に短いためその多量を長時間にわたって投与しなければならないという制約があり、このため出血傾向の副作用をしばしば伴うことが報告されている。このため半減期を延長させるべく、あるいは血中に存在するインヒビターによる阻害を受けないタイプのtPAムテインが作製された。しかし、このようなtPAムテインは一般的に元の非修飾型tPAに比べてフィブリン親和能が小さいため血栓への選択性が低下したり、あるいは酵素活性そのものが低下することが分っている。また抗体ターゲティング化血栓溶解剤については血栓溶解作用物質の血栓溶解能を増強させるだけでなく、その血中半減期をも延長させる効果を有しているが、同時に血栓溶解剤のもう副作用【フィブリノーゲン分解能および α_2 -アンチプラスミン（以下、 α_2 -APと略記することがある）消費能】を増大させることはある程度避けられないことが分かっている。

【0004】

【課題を解決するための手段】本発明者らは、上記のtPAムテインで見られた血栓親和能の低下や、あるいは抗体ターゲティング化血栓溶解剤で見られた副作用の増大という問題点を解決するため種々の検討を重ね、tPAムテインをbsMoAbに免疫結合させ、血栓へのターゲティングを実施することにより、血中半減期の延長、血栓親和能の増大、さらには副作用の軽減をもたらし得ることを見出した。すなわち、本発明は二重特異性の一方が血栓に対するものであり、他方がF、EおよびK1ドメインを欠失するtPAムテインに対するものであるbsMoAbであり、さらにはこのbsMoAbに

該tPAムテインを免疫結合させてなる血栓溶解剤を提供するものである。本発明の二重特異性抗体は、血栓がフィブリンあるいは活性化血小板であるものに結合できるものであり、また該抗体は少なくとも可変領域を含有し、かつ重鎖定常領域ドメイン2および3を欠失したものであることができる。

【0005】tPAムテインについて説明すると、tPAは図1に示すようにFINGER(F), EGF(E), KRINGLE1(K1), KRINGLE2(K2)およびPROTEASE(P)部分から構成されている。上記tPAムテインとしてはE, F, K1部分が欠失した後述の参考例16に記載のtPA-1や、その内のP部分のR^{298' 299}がE^{298' 299}に置換された後述の参考例19に記載のtPA-6'等が挙げられる。また抗体分子は図2に示すように、抗原との結合に関わる可変(V)領域と、抗原結合には関与しないが、他の生物学的機能、例えば補体との結合あるいは抗体レセプターとの結合に関与する定常(C)領域がある。抗原結合に関わる機能のみを必要とする場合には可変領域のみで構成される分子で十分であり、最近の遺伝子組換型抗体の中にはこのような分子も作製されている。古くから採用されている方法は、抗体分子をババインあるいはペプシンのような消化酵素を用いて限定的な部分加水分解処理に供し、図2に示した個所でペプチドを切断し重(H)鎖定常領域ドメイン2および3(以下それぞれCH₁およびCH₂と略記することがある)を欠失したFabあるいはFab'を作製する方法である。このようにして得られた抗体断片は幾つかの抗体機能を失うが、少なくとも抗原結合能はほぼ100%保持されているのが通例であり、本発明においてもこのような断片を用いることができる。

【0006】本発明で用いられる、tPAムテインとして、F、E、K1の全ドメインを欠失し、K2、プロテアーゼ(P)の両ドメインからなるtPAムテイン(アミノ酸残基番号174-527、ただし174~179の一部ないしは全部のアミノ酸残基を欠失してもよい)、および該tPAムテインの更なるムテインが挙げられる(以下、両者をあわせてFEK1欠失tPAムテインと略記することがある)。また、この更なるムテインとしては、本来、元のペプチドあるいは蛋白質のアミノ酸配列が変異したものが挙げられ、したがって該変異としては、構成アミノ酸の欠損、他のアミノ酸への置換が挙げられる。該構成アミノ酸の欠損としては、FEK1欠失tPAムテイン構成アミノ酸の少なくとも1個が欠損しているものが挙げられる。該他のアミノ酸への置換としては、FEK1欠失tPAムテイン構成アミノ酸の少なくとも1個が別のアミノ酸で置換されているものが挙げられる。少なくとも1個の構成アミノ酸が欠損しているFEK1欠失tPAムテインにおいて欠損している構成アミノ酸の数としては、FEK1欠失tPAムテインの有する特

徴を失わない限り何個でもよい。該欠損している構成アミノ酸の例としては、tPAのアミノ酸残基番号296から302のアミノ酸残基などが挙げられる。

【0007】少なくとも1個の構成アミノ酸が別のアミノ酸で置換されているFEK1欠失tPAムテインにおいて置換されるアミノ酸の数としては、FEK1欠失tPAムテインの特徴を失わない限り何個でもよい。置換されるアミノ酸部位としては、アミノ酸残基番号296から304の間のアミノ酸残基の一部ないし全部を別のアミノ酸残基に置換してなるものや、アミノ酸残基番号296から302の領域の一部ないし全部を欠失させ、かつアミノ酸残基番号296から304の領域の一部のアミノ酸残基を別のアミノ酸残基に置換してなるものがその例として挙げられる。置換される前の構成アミノ酸の例としては、例えばシステイン、アスパラギン酸、アルギニンなどが挙げられる。置換される前の構成アミノ酸がシステインである場合には、置換されたアミノ酸としては、たとえば中性アミノ酸が好ましい。該中性アミノ酸の具体例としては、たとえば、グリシン、バリン、アラニン、ロイシン、イソロイシン、チロシン、フェニルアラニン、ヒスチジン、トリプトファン、セリン、スレオニン、メチオニンなどが挙げられる。特に、セリン、スレオニンが好ましい。

【0008】置換される前の構成アミノ酸がシステイン以外のものである場合には、置換された別のアミノ酸としては、たとえば、アミノ酸の親水性、疎水性あるいは電荷の点で、置換される前のアミノ酸とは異なる性質をもつものを選ぶ。具体的には置換される前のアミノ酸がアスパラギン酸の場合には、置換されたあとのアミノ酸としてアスパラギン、スレオニン、バリン、フェニルアラニン、アルギニンなどが挙げられるが、特にアスパラギン、アルギニンが好ましい。置換される前のアミノ酸がアルギニンの場合には置換されたあとのアミノ酸としてグルタミン、グルタミン酸、スレオニン、ロイシン、フェニルアラニン、アスパラギン、アスパラギン酸が挙げられるが、特にグルタミン酸あるいはヒスチジンが好ましい。置換される前のアミノ酸がグルタミン酸の場合には、置換された後のアミノ酸としてチロシンが好ましい。本発明のムテインは、上記した欠損、置換が組み合わされたものでもよい。

【0009】本発明のムテインを製造するためには、従来の組換えDNA技術に加え、特定部位指向性変異誘発技術(Site-directed mutagenesis)が採用される。該技術は周知であり、アール・エフ・レイサー(Lather, R. F.)及びジェイ・ピー・レコック(Lecoq, J. P.)、ジェネティック・エンジニアリング(Genetic Engineering)、アカデミックプレス社(1983年)第31-50頁、に示されている。オリゴヌクレオチドに指示された変異誘発はエム・スミス(Smith, M.)及びエス・ギラム(Gillam, S.), ジェネティック・エンジニアリング:原理と方

法、ブレナムプレス社(1981年)3巻1-32頁に示されている。

【0010】本発明のムテインをコードする構造遺伝子を製造するためには、たとえば、(1) tPAの構造遺伝子の1本鎖からなる1本鎖DNAを突然変異オリゴヌクレオチドプライマーと雑種形成させる、(2) DNAポリメラーゼによりプライマーを伸長させ、突然変異性ヘテロ二量体(heteroduplex)を形成させる、及び(3)この突然変異性ヘテロ二量体を複製する、といった方法がある。

【0011】本発明で用いられる抗フィブリノーゲン抗体産生ハイブリドーマの作製にあたっては、該ハイブリドーマがフィブリノーゲンに特異的で実質的にフィブリノーゲンと結合しないMoAbを産生するものであればいずれのものでもよい。例えばかかるフィブリノーゲン特異抗体は、フィブリノーゲンが分解されて生ずるフィブリノーゲンの、 α 鎖N末端フラグメントペプチドあるいは β 鎖N末端フラグメントペプチドを免疫原として用いることにより作製される【K. Y. Huiら:サイエンス(Science), 222, 1129 (1983);特開昭63-93800号公報】。また、フィブリノーゲンは哺乳動物のものであればいずれでもよいが、好ましくはヒト・フィブリノーゲンが挙げられ、とりわけヒト・フィブリノーゲンの β 鎖N末端部に相当するペプチドが用いられる。これにキャリア蛋白を結合させて、動物(例、ウサギ、ラット、マウス、モルモットなど)を免疫し抗体産生細胞を得る。次いで免疫動物より採取したこれらの抗体産生細胞、例えば脾臓細胞やリンパ節細胞などを骨髓腫細胞と融合し、得られるハイブリドーマの中から実質的にフィブリノーゲンに反応せずフィブリノーゲンに特異的に結合する抗体産生細胞をスクリーニングする。上記のヒト・フィブリノーゲン β 鎖N末端ペプチドとして、次のときアミノ酸配列を有するものが特に好ましく用いられる。

H-Gly-His-Arg-Pro-Leu-Asp-Lys-R-Cys-OH

【式中、RはLys-Arg-Glu-Gluで示されるペプチド又はその一部を示す】【配列番号1】。C末端のCysはキャリア蛋白との化学結合用のリンカーパートに用いられる。すなわちキャリア蛋白を予め、例えばN-(γ -マレイミドブチリルオキシサクシニミド)(以下、GMBSと略記することがある)でマレイミド化あるいはN-サクシニミジル-3-(2-ビリジルジチオ)プロピオネート(以下、SPDPと略記することがある)でジチオビリジル化することにより、上記ペプチドのC末端CysのSH基を介してキャリア蛋白に化学結合させることができある。

【0012】さらにフィブリノーゲン特異性を確実なものとするために、T. Hamaokaらが報告したD-グルタミン酸とD-リジンの共重合体(以下、D-GLと略記することがある)を用いて、フィブリノーゲン抗原免疫動物にフィブリノーゲン寛容状態を惹起させ、フィブリノーゲン特異抗体産生細胞をより高頻度で得る方法も好ましく用いられる。

【T. Hamaokaら:ジャーナル・オブ・エクスperimental・メディシン(J. Exp. Med.), 139, 1446 (1974); K. Tateishiら:ジャーナル・オブ・イムノロジカル・メソッズ(J. Immunol. Methods), 47, 249 (1981)】。この方法では、免疫原として例えば上記のヒト・フィブリノーゲン β 鎖N末端ペプチドにキャリア蛋白を結合させたものが、寛容原として例えば次のときアミノ酸配列を有するペプチドにD-GLを結合させたものが用いられる。

10 H-Phe-Phe-Ser-Ala-Arg-Gly-His-Arg-Pro-Leu-Asp-Lys-R-OH

【式中RはLys-Arg-Glu-Gluで示されるペプチド又はその一部を示す】【配列番号2】。

【0013】また本発明で用いられる抗活性化血小板MoAb産生ハイブリドーマの作製にあたっては、該ハイブリドーマが活性化血小板に特異的かつ実質的に非刺激血小板と結合しないMoAbを産生するものであれば、いずれのものでもよい。例えばかかる活性化血小板特異MoAbはトロンビンで活性化された血小板(好ましくはヒト血小板)を免疫原として用いることにより作製される【C. L. Bermanら:ジャーナル・オブ・クリニカル・インベスティゲーション(J. Clin. Invest.), 78, 130 (1986); N. Akamatsuら:スロンボーシス・アンド・ヘモスタシス(Thromb. Haemostasis), 62, 250 (1989)】。また、用いる血小板は哺乳動物のものであればいずれでもよいが、好ましくはヒト血小板が挙げられる。この活性化血小板を動物(例、ウサギ、ラット、マウス、モルモットなど)に免疫し抗体産生細胞を得、次いで免疫動物より採取したこれらの抗体産生細胞、例えば脾臓細胞やリンパ節細胞などを骨髓腫細胞と融合する。

得られるハイブリドーマの中から実質的に非刺激血小板に反応せず活性化血小板に特異的に結合する抗体産生細胞をスクリーニングすることによって、目的とする抗活性化血小板MoAb産生ハイブリドーマを取得することができる。さらにまた本発明における抗tPAムテインMoAb産生ハイブリドーマの作製については、前述したtPAムテイン、あるいは元の非修飾型のtPAそのものを常法に従い動物に免疫し、得られる抗体産生細胞を骨髓腫細胞などと融合させる方法が用いられる。動物を免疫し、得られる抗体産生細胞を骨髓腫細胞などと融合させ、抗体産生ハイブリドーマを得る方法については、抗フィブリノーゲン抗体産生ハイブリドーマあるいは抗活性化血小板抗体産生ハイブリドーマを得る方法と同様な操作が用いられる。

【0014】免疫動物としては、例えばウサギ、ラット、マウス、モルモットなどが用いられるが、MoAb製造の場合にはマウスが特に好ましく用いられる。接種方法としては、通常実施される方法に従えばよく、例えば活性化血小板に特異的な抗体を作製する場合にはマウス

に1回 10^6 ~ 10^{10} 個、好ましくは 0.5 ~ 2×10^9 個の洗浄ヒト血小板を生理食塩水、ヘペス緩衝液あるいはリン酸食塩緩衝液（以下、PBSと略記することがある）に懸濁して、トロンビンで活性化後腹腔内に10~14日毎に3~8回接種する方法がとられる。ヒトフィブリンやtPAムテインに特異的な抗体を作製する場合には、マウスに1回 1 ~ $100 \mu\text{g}$ 、好ましくは 1 ~ $2.5 \mu\text{g}$ の抗原蛋白質を等容量（ 0.1 ml ）の生理食塩水およびフロントの完全アジュバンドで乳化して、背部、腹部の皮下あるいは腹腔内に2~3週毎に3~6回接種する方法がとられる。これらの免疫動物、例えばマウスから抗体価の高い個体を選び、最終免疫3~5日後に脾臓およびあるいはリンパ節を採取し、それらに含まれる抗体産生細胞を骨髓腫細胞と融合させる。融合操作は既知の方法に従い実施でき、融合促進剤としてはポリエチレングリコール（以下、PEGと略記することがある）やセンダイ・ウィルスなどが挙げられるが、好ましくはPEGが用いられる。骨髓腫細胞としてはNS-1、P3U1、SP2/0など、特にNS-1やP3U1が好ましく用いられる。例えば脾臓細胞と骨髓腫細胞との好ましい比率は $1:1$ ~ $10:1$ で、これに分子量 $1,000$ ~ $9,000$ のPEGが 10 ~ 80% の濃度で添加され、 20 ~ 37°C 、好ましくは 30 ~ 37°C で3~10分インキュベートするのが良い。

【0015】抗フィブリン抗体産生ハイブリドーマのスクリーニングには種々の方法が使用できる。例えば、マイクロプレートにフィブリノーゲンを吸着させたのち、トロンビンを作用させフィブリノーゲンをフィブリンに変換する。次いで過剰のフィブリノーゲン存在下でハイブリドーマ培養上清をフィブリン固定マイクロプレートに添加し、プレートに結合した抗フィブリン特異抗体を検出する酵素免疫測定法（以下、EIAと略記することがある）により培養上清中の抗体価を測定する。HAT（ヒポキサンチン・アミノブテリン・チミジン）添加培地で選別、育種された抗体活性陽性のハイブリドーマは直ちにクローニングに供されるが、通常これは限界希釈法などで容易に実施される。クローニングされたハイブリドーマ培養上清の抗体価を上記の方法で測定し、安定的に力価の高い抗体を産生するハイブリドーマを選択し、目的とするモノクローナルな抗フィブリン特異抗体産生ハイブリドーマを取得することができる。以上のような製造法に従って、作製した抗フィブリン抗体産生ハイブリドーマの例として、後述の参考例8および実施例1に示したFIB1-11, FIB2-11およびFTB2-133が挙げられる。抗活性化血小板MoAb産生ハイブリドーマのスクリーニングについても種々の方法が使用できる。例えば、マイクロプレートに非刺激血小板あるいはトロンビン活性化血小板を結合させ、1%ホルマリンで固定化して固相抗原として使用する。これにハイブリドーマ培養上清を添加し、プレートに結合した抗活

性化血小板抗体を酵素標識第2抗体で検出するEIAにより培養上清中の抗体価を測定し、非刺激および活性化血小板との結合の差の大きなものを選択する。例えば、HAT添加培地で選別、育種された抗体活性陽性のハイブリドーマは直ちにクローニングに供されるが、通常これは限界希釈法などで容易に実施される。クローニングされたハイブリドーマ培養上清の抗体価を上記の方法で測定し、安定的に力価の高い抗体を産生するハイブリドーマを選択し、目的とするモノクローナルな抗活性化血小板特異抗体産生ハイブリドーマを取得することができる。以上のような製造法に従って作製した抗活性化血小板MoAb産生ハイブリドーマの例として、後述の参考例9に示したマウス・ハイブリドーマ2T60が挙げられる。

【0016】またtPAムテインに対するMoAbを産生するハイブリドーマのスクリーニングは、tPAムテインあるいは非修飾型tPAを吸着させたマイクロプレートを用いるEIAで簡便に実施できる。クローニングも上記した常法に従って実施し、目的の抗tPAムテイン20 MoAb産生ハイブリドーマを取得できる。以上のような製造法に従って作製した抗tPAムテインMoAb産生ハイブリドーマの例として、後述の参考例10に示したマウス・ハイブリドーマTPA1-41が挙げられる。

【0017】本発明の二重特異性を有するハイブリッドMoAbを産生するポリドーマの作製には幾つかの手法があり【例、新本洋士ら：蛋白質・核酸・酵素、33, 217(1988)など】、いずれの方法を用いてもよいが例えば、①上記のHAT抵抗性の抗tPAムテイン抗体産生ハイブリドーマを、5-ブロモデオキシリジン（以下、BrdUと略記することができる）添加の培養液に段階的に馴化させ、チミジンキナーゼ欠損株をクローニングしHAT感受性とする。同様にHAT抵抗性の抗フィブリンあるいは抗活性化血小板特異抗体産生ハイブリドーマを8-アザグアニン（以下、AZGと略記することができる）耐性とし、ヒポキサンチングアニン-ホスホリボシルトランスフェラーゼ欠損株をクローニングしHAT感受性とする。次いで常法に従い両者を融合して得られるテトラオーマをHAT添加培地で選別後、血栓およびtPAムテインの両者に結合能を有するハイブリッドMo40 Abを分泌するテトラオーマをクローニングする、②抗フィブリンあるいは抗活性化血小板特異抗体産生ハイブリドーマをフルオレセイン・イソチオシアネート（以下、FITCと略記することができる）で標識し、もう一方の抗tPAムテイン抗体産生ハイブリドーマをテトラメチル・ロダミン・イソチオシアネート（以下、TRITCと略記することができる）で標識後、常法に従い両者を融合する。得られた細胞懸濁液をフルオレセイン・アクティベイティッド・セルソーター（以下、FACSと略記することができる）に供し、FITCの緑色およびTRITCの赤色の蛍光を同時に有するテトラオーマを選別・

クローニングするなどの方法が挙げられる。また両親株のマーカーを全く逆にして使用し、テトラオーマを選別・クローニングすることも可能である、③tPAムテインを免疫したマウスより脾臓細胞を採取し、一方でHAT感受性とした抗フィブリンあるいは抗活性化血小板MoAb産生ハイブリドーマとこの脾臓細胞とを融合させ、HAT添加培地で選別しトリオーマを作成する。次いで血栓およびtPAムテインの両者に結合能を有するハイブリッドMoAbを分泌するトリオーマをクローニングする、などの方法がある。

【0018】これらの操作における細胞融合に当ってはセンダイウイルス、PEGなどの融合促進剤あるいは電気刺激などの方法が用いられる。好ましくはPEGが用いられ、以下にその一例を挙げるが、もちろんこの方法に限定されるものではない。すなわち、分子量約1,000~9,000、濃度約10~80%等のPEGが用いられ、処理時間は約0.5~30分であるが、好ましい条件の一例として、約35~55%のPEG 6,000を約4~10分間、37°Cで細胞と接触させ、効率よく融合させることができる。ポリドーマの選択は、上記のHAT添加培地などで実施できるが、このため8-AZG、6-チオグアニン(6-TG)あるいは5-BrdUなどの薬剤馴化法により、それぞれの薬物耐性株が取得される。また新しいマーカーの融合細胞への導入により、種々の選択培地が用いられる。このような例として、ネオマイシンやハイグロマイシンB添加培地などが挙げられる【B. Sugdenら:モレキュラー・アンド・セラーラー・バイオロジー(Mol. Cell. Biol.), 5, 410 (1985)】。さらに前記したように、異った蛍光色素で標識したハイブリドーマを融合し、FACSで二重標識されたハイブリッド・ハイブリドーマをソーティングする方法もある【L. Karawajewら:ジャーナル・オブ・イムノロジカル・メソッズ(J. Immunol. Methods), 96, 265 (1987)】。

【0019】ハイブリッド抗体産生ポリドーマのスクリーニングには種々の方法が使用できる。例えば、①前述した抗フィブリンあるいは抗活性化血小板特異抗体産生ハイブリドーマと抗tPAムテイン抗体産生ハイブリドーマのスクリーニングのためのEIA併用、②フィブリン結合あるいは活性化血小板結合マイクロプレートに被検培養上清を添加し、次にHRP標識したtPAムテインもしくは非修飾型tPAを加えて二重特異性を有するハイブリッド抗体検出のためのEIA;あるいは抗フィブリンあるいは抗活性化血小板特異抗体と異なるサブクラスに属するtPAムテインに対する抗体を用いる場合は、③フィブリン結合あるいは活性化血小板結合マイクロプレートに被検培養上清を添加し、次にHRP標識した該抗マウスIgGサブクラス特異抗体を加えて二重特異性抗体を検出するEIA、およびこれらの変法などを適宜組み合せて用いることができる。ハイブリッド抗体活

性陽性のポリドーマは直ちにクローニングに供されるが、これは通常限界希釈法などで容易に実施される。クローニングされたポリドーマの培養上清については、上記の方法でその抗体価を測定し、安定的に力価の高い抗体を産生するポリドーマを選択することにより、目的とするモノクローナルなハイブリッド抗体産生ポリドーマを取得することができる。

【0020】上記した本発明のポリドーマの培養は通常、液体培地中、または動物の腹腔内(例えば、マウス等哺乳動物の腹腔内)で公知の方法により実施できる。培養液および腹水中の抗体の精製については公知の生化学的手法を組み合わせて用いることによりできる。例えば、細胞培養液もしくは腹水を遠心分離し、上清を取り出し、塩析(通常は硫酸アンモニウムもしくは硫酸ナトリウムを用いる)を実施する。得られたタンパク沈殿物を適当な溶液に溶解し、透析後カラムクロマトグラフィー(イオン交換カラム、ゲルろ過カラム、プロテインAカラム、ヒドロキシアバタイトカラム、ABxカラム、疎水担体カラムあるいは抗原結合カラム等)に付し、目的とする抗体を分離精製することができる。以上のような分離精製操作により、例えば1リットルの培養上清からタンパク重量比で90%以上の純度のハイブリッドMoAbを約1~5mg得ることができる。また、20mlの腹水液からは同様の抗体が3~10mg得られる。以上のような製造法に従って作製した抗フィブリン-抗tPAムテイン二重特異性抗体産生テトラオーマの例として実施例3に示したFT2-14がトリオーマの例として実施例2に示したTAF1-42、TAF1-79およびTAF1-228が挙げられる。本発明の二重特異性抗体がマウス抗体由来のものである場合には、該蛋白質の超可変領域以外の領域、例えば定常領域あるいはフレーム・ワーク領域を、遺伝子操作技術によりヒト抗体由来のものに変換し【Z. Steplewskiら:プロシーディングズ・オブ・ナショナル・アカデミー・サイエンス ユーエスエー(Proc. Natl. Acad. Sci. USA), 85, 4852 (1988); L. Riechmannら:ネチャー(Nature), 332, 323 (1988)】、マウス-ヒト・キメラ型あるいはヒューマナイズした抗体を作製することもできる。かかるヒト型化抗体はヒトへの投与に際し、抗原性が小さいため有利に用いられる。

【0021】本発明の二重特異性抗体を作製するには、上記のハイブリッド・ハイブリドーマ法が望ましいが、以下のような化学結合法も用いられる。2種のMoAbを化学的に結合させるために、抗体分子中に存在している置換基、例えばアミノ基、カルボキシル基、ヒドロキシル基またはスルフヒドリル基などを利用することができる。例えば、(1)一方の抗体の反応性アミノ基と他方の反応性カルボキシル基とを水溶性カルボジイミド試薬(例、1-エチル-3-(3-ジメチルアミノ)プロピル)カルボジイミド、1-シクロヘキシル-3-(2

一モルホリノエチル) -カルボジイミド- p -トルエンスルホネートなど)を用いて水性溶媒中で脱水縮合させる、(2)一方の抗体の反応性アミノ基をN-ヒドロキシスクシミドの活性エステル〔例、p-マレイミドメチルシクロヘキサン-1-カルボキシル-N-ヒドロキシスクシミドエステル、N-(ε-マレイミドカブロイロキシ)スクシミドエステルあるいはGMB Sなど〕と反応させマレイミド化したのち、i)他方の抗体をジオースレイトール(DTT)で還元した抗体、あるいはii)他方の抗体にN-スクシミジル-3-(2-ビリジルジチオ)プロピオネート(SPD P)でスルフヒドリル基を導入した抗体、あるいはiii)他方の抗体をペブシン処理後還元して得られるF(ab')2分画のスルフヒドリル基とチオエーテル結合させる、(3)2種の抗体双方の反応性アミノ基をスクシジアルデヒドやグルタルアルデヒドなどのシアルデヒド試薬を用いて結合させる。

(4)2種の抗体をDTTで還元あるいはSPDPでスルフヒドリル基を導入し、再酸化によりヘテロダイマーを作製する、(5)2種の抗体をいずれもペブシン処理後還元し、F(ab')2としたのち再酸化しF(ab')2へテロダイマーを作製する、などの方法がある。またこれらの方法を種々組み合わせて、2種の抗体活性をできるだけ損なわずに効率良く目的のヘテロダイメリックな二重特異性抗体を作製する報告があり〔M. J. Glennieら:ジャーナル・オブ・イムノロジー(J. Immunol.), 139, 2367 (1987);北川常広:有機合成化学、42, 283 (1984)〕、本発明の二重特異性MoAbの作製に利用できる。

【0022】以上のような結合反応終了後、二重特異性抗体結合物はセファデックスG100もしくはG200、セファロース6Bもしくは4B、ウルトロゲルAcA44もしくは34、セファクリルS200などのゲルろ過クロマトグラフィーにより精製・分取できる。あるいは抗原結合カラムを用いるアフィニティーコロマトグラフィーを組み合わせることにより選択的な分取も可能である。

【0023】以上のようにして得られた二重特異性を有するハイブリッドMoAbは蛋白質として均一であり、蛋白分解酵素(ペプシン、ババインなど)処理などにより、活性化血小板あるいはフィブリンおよびtPAムテインに対する結合能を保持するF(ab')2断片などを得ることができる。本発明の二重特異性MoAbあるいはtPAムテインと該二重特異性MoAbとから作製される選択的な血栓溶解蛋白複合体を用いる血栓溶解治療法においては、幾つかの方法が用いられる。例えば、①本発明の二重特異性MoAbを予め血栓性疾患患者に投与し、患者体内に形成された血栓に結合させるべく十分な時間経過後に、tPAムテインを投与する、②該二重特異性MoAbとtPAムテインとを同時に血栓性疾患患者に投与する。あるいは③予め該二重特異性MoAbとtPAムテインとを反応させ、たとえば免疫結合させ、未反応のt

PAムテインを分離後、得られた選択的血栓溶解蛋白複合体、好ましくは該bSMoAbとtPAムテインの比率が1:1であるような選択的血栓溶解蛋白複合体を、血栓性疾患患者に投与するなどの方法が挙げられる。

【0024】本発明の二重特異性MoAbあるいはさらにtPAムテインを免疫結合させてなる血栓溶解剤は、必要により例えばメンブレンフィルター等による過除菌操作の後に、それ自体あるいは適宜の薬理学的に許容され得る担体、賦形剤、希釈剤などと混合し、注射剤などとして製剤化して、哺乳動物(例、マウス、ラット、ネコ、イヌ、ブタ、ウシ、サル、ヒトなど)に投与し、例えば心筋梗塞、末梢動・静脈閉塞症、網膜動・静脈閉塞症、脳梗塞、肺塞栓症などの血栓・閉塞性疾患の治療に用いることが可能である。本発明の血栓溶解剤の投与量は、対象となる疾患、症状あるいは投与ルートなどによって異なるが、例えば心筋梗塞の成人患者に静脈内投与する場合、二重特異性MoAbとして1日当り約0.02~1mg/kg好ましくは約0.04~0.4mg/kg、tPAムテインでは一般的には約0.001~0.5mg/kg、好ましくは約0.002~0.1mg/kg、中でも約0.004~0.04mg/kgが好ましい。

【0025】

【作用】以上のような方法で、標的血栓部位に対して特異的に結合可能であり、実質的にフィブリノーゲンや非刺激血小板と結合しない本発明の二重特異性MoAbとtPAムテインとを用いることにより、副作用を伴うことなく選択的かつ効率的に血栓を溶解、除去できる。

【0026】本発明明細書および図面において、塩基やアミノ酸などを略号で表示する場合、IUPAC-IUB Commission on Biochemical Nomenclatureによる略号あるいは当該分野における慣用略号に基づくものであり、その例を下記する。また、アミノ酸に関し光学異性体がありうる場合は、特に明示しなければL-体を示すものとする。

DNA	: デオキシリボ核酸
cDNA	: 相補的デオキシリボ核酸
A	: アデニン
T	: チミン
G	: グアニン
C	: シトシン
RNA	: リボ核酸
dATP	: デオキシアデノシン三リン酸
dTTP	: デオキシチミン三リン酸
dGTP	: デオキシグアノシン三リン酸
dCTP	: デオキシチジン三リン酸
ATP	: アデノシン三リン酸
Tdr	: チミジン
EDTA	: エチレンジアミン四酢酸
SDS	: ドデシル硫酸ナトリウム
Gly (G)	: グリシン

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A l a (A) : アラニン
 V a l (V) : バリン
 L e u (L) : ロイシン
 I l e (I) : イソロイシン
 S e r (S) : セリン
 T h r (T) : スレオニン
 C y s (C) : システイン
 M e t (M) : メチオニン
 G l u (E) : グルタミン酸
 A s p (D) : アスパラギン酸
 L y s (K) : リジン
 A r g (R) : アルギニン
 H i s (H) : ヒスチジン

* P h e (F) : フェニルアラニン
 T y r (Y) : チロシン
 T r p (W) : トリプトファン
 P r o (P) : プロリン
 A s n (N) : アスパラギン
 G l n (Q) : グルタミン。
 【0027】

【実施例】以下に参考例および実施例により本発明を具体的に説明するが、これらが本発明の範囲を制限するものでないことは言うまでもない。なお、参考例および実施例で用いられている動物細胞および微生物は、以下の表に示すように寄託が行なわれている。

*

動物細胞および微生物名	(IFO)	(FRI)
IFO No.	FERM No.	
マウスハイブリドーマ	50174	BP-2081
FIB 1-11	(1988.9.21)	(1988.10.4)
マウスハイブリドーマ	50175	BP-2082
FIB 2-11	(1988.9.21)	(1988.10.4)
マウスハイブリドーマ	50178	BP-2085
TPA 1-41	(1988.9.21)	(1988.10.4)
マウスハイブリドーマ	50211	BP-2623
2T60	(1989.9.27)	(1989.10.4)
マウスハイブリドーマ	50332	BP-3455
FTB 2-133	(1991.6.7)	(1991.6.18)
マウスハイブリッド・ハイブリドーマ	50180	BP-2158
(テトラオーマ) FT2-14	(1988.11.8)	(1988.11.25)
マウスハイブリッド・ハイブリドーマ	50333	BP-3456
(トリオーマ) TAF 1-42	(1991.6.7)	(1991.6.18)
マウスハイブリッド・ハイブリドーマ	50334	BP-3457
(トリオーマ) TAF 1-79	(1991.6.7)	(1991.6.18)
マウスハイブリッド・ハイブリドーマ	50335	BP-3458
(トリオーマ) TAF 1-228	(1991.6.7)	(1991.6.18)
Escherichia coli MM294(DE3)/pLysS, pTB1133	IFO 15031	FERM BP-2882
	(1990.4.17)	(1990.5.1)
Escherichia coli MM294(DE3)/pLysS, pTB1277	IFO 15116	FERM BP-3199
	(1990.12.6)	(1990.12.13)

IFO:財団法人発酵研究所（大阪市淀川区十三本町2丁目
17番85号）

FRI:通商産業省微生物工業技術研究所（茨城県つくば市
東1丁目1番3号）

【0028】

【参考例1】 抗フィブリノーゲン抗体測定用E I A
3.3M尿素、0.01% EDTA含有リン酸食塩緩衝液 (PBS, pH 7.3) に溶解したヒト・フィブリノーゲン・モノマー溶液 1 mg/ml を、96穴マイクロプレートに 50 μlずつ分注し 4°Cで一夜放置後、2%カゼイン、0.01%チメロサール含有 PBS 150 μl を添加して感作プレートを作製した。次に 100 単位/ml へパリン、3 mM フェニルメチルスルホニルフルオリド含有 P

BS に溶解したヒト・フィブリノーゲン溶液 10 mg/ml を、等量の被検ハイブリドーマ培養上清と混じ、室温で 30 分間反応後、その 100 μl を上記のフィブリノーゲン作プレートに添加し室温で 2 時間反応させた。0.05% Tween20 含有 PBS (PBS-Tw) でプレートを十分に洗浄後、ホースラッディッシュ・ベルオキシダーゼ (HRP) 標識ウサギ抗マウス IgG 抗体を添加し、さらに室温で 2 時間反応させた。洗浄後、酵素基質としてオルソフェニレンジアミンおよび H2O2 を含有する 0.1 M クエン酸緩衝液を各ウェルに加え、室温で酵素反応を実施した。1 N 硫酸で反応停止後、マルチスキャナー (フロー社製) を用いて波長 492 nm で発色色素量を測定した。

50

【0029】

【参考例2】抗血小板抗体測定用EIA

①固定化血小板の作製

クエン酸採血したヒト新鮮血から遠心分離法により多血小板血漿を取得し、ADP分解酵素を含むタイロードーベース緩衝液(pH 6.5)で洗浄した。この洗浄血小板を 2×10^7 個/ウェルでマイクロプレートに播き、トロンビン(0.2ユニット/ml)で活性化したのち遠心した。次いで2%ホルマリンで固定化後、5%牛血清アルブミン(以下、BSAと略記することがある)含有PBSでブロッキングして活性化血小板プレートを作製した。非刺激血小板プレートは上記の操作中、トロンビン活性化操作を省略して作製した。

②EIA操作法

ハイブリドーマ培養上清100μlを血小板プレートに添加し、室温で3時間反応後PBS-Twで洗浄、HRP標識ウサギ抗マウスIgG抗体を添加し、さらに室温で2時間反応させた。洗浄後、固相に結合したHRP活性を参考例1に示した方法で測定した。

【0030】

【参考例3】抗tPA抗体測定用EIA

市販の1本鎮tPA(中央科学工業K.K.販売)5μg/ml溶液を96穴マイクロプレートに100μlずつ分注し4°Cで一夜放置後、2%カゼイン、0.01%チメロサール含有PBS150μlを添加して感作プレートを作製した。上記の液を除去しPBS-Twで洗浄後、被検ハイブリドーマ培養上清100μlを添加し室温で2時間反応させた。以下、参考例1に記載の方法で酵素反応を実施し抗体価を測定した。

【0031】

【参考例4】抗tPAムテイン抗体測定用EIA

参考例3に記載のtPAの代りに参考例19に記載のtPA-6'を使用し、tPA-6'感作プレートを作製後、同様の方法で抗tPAムテイン抗体価を測定した。

【0032】

【参考例5】抗ヒト・フィブリノーゲン抗体測定用EIA

参考例3に記載のtPAの代りに市販のヒト・フィブリノーゲン(和光純薬販売)を使用し、ヒト・フィブリノーゲン感作プレートを作製後、同様の方法で抗ヒト・フィブリノーゲン抗体活性を測定した。

【0033】

【参考例6】抗フィブリン-抗tPAムテイン・ハイブリッド抗体測定用EIA

参考例4で作成したtPA-6'感作プレートにハイブリッド抗体含有検液を添加し、室温で2時間反応させた。PBS-Twで洗浄後、ビオチン標識した参考例8-①に記載のヒト・フィブリンβ鎖N末端ペプチド(1-11)-BSA複合体を添加し、さらに室温で2時間反応させた。次にアビシン-HRP複合体を添加し室温

で1時間反応後、固相に結合したHRP活性を参考例1に示した方法で測定した。

【0034】

【参考例7】フィブリン溶解反応中和試験

tPA溶液(最終濃度20ng/ml)に被検ハイブリドーマ培養上清希釈液を添加し、37°Cで1時間反応後反応混液をフィブリン・アガロース・プレートの1ウェル当たり5μl注入した。37°Cで2~6時間後にフィブリンの溶解斑(直径)を測定し、tPAの酵素活性に対するハイブリドーマ培養上清に含まれるMoAbの中和能を測定した。

【0035】

【参考例8】マウス抗ヒトフィブリン・モノクロナル抗体産生ハイブリドーマの作製

①免疫原の調製

公知の固相合成法によりペプチド合成機(アブライド・システム、モデル430A型)を用いて作製された次式のヒト・フィブリンβ鎖N末端ペプチド(1-11)-Cys 3.3mgを、予めGMB Sでマレイミド化したBSA(BSA1モル当たり13モルのマレイミド基を導入)12mg/2ml水溶液に加え30°Cで1時間反応させ、ヒト・フィブリンβ鎖N末端ペプチド(1-11)-BSA複合体を得た。次いで生理食塩水で3回透析後(3リットル×3)、凍結保存し免疫原として用いた。

H-Gly-His-Arg-Pro-Leu-Asp-Lys-Lys-Arg-Glu-Glu-Cys-OH(12-mer, 配列番号1)

②免疫

ペプチド-BSA複合体1mg/ml生理食塩水溶液に等量のフロイント完全アジュバントを加え、マウス(♀, n=10:0.1mg/0.2ml/マウス)の背部および腹部皮下への免疫を開始した。追加免疫は免疫原に等量のフロイント不完全アジュバントを加えて、2~3週毎に5回接種し実施した。

③細胞融合

最終免疫後3日で脾臓を摘出し、脾臓細胞懸濁液を常法により調製した(約10⁸個)。次いでマウス骨髄腫細胞(P3U1)2×10⁷個を添加し、PEG6000を用いてケーラーとミルスタンの方法[Nature, 256, 495 (1975)]に準じて細胞融合に供した。融合終了後、細胞混液をHAT培地中に懸濁し、10日間培養した。以後は、親細胞の選択が終了次第、HAT培地からアミノブテリンを除いたHT培地に代え培養を続けた。

【0036】④ハイブリドーマの選択およびクローニング

固相にヒト・フィブリノーゲンおよびヒト・フィブリン・モノマーを吸着させたマイクロプレートを用いる参考例5および1に記載のEIAでハイブリドーマ培養上清の抗体価を測定した。融合10日から20日後でハイブリドーマの出現を認め、かつヒト・フィブリンに特異結

合する抗体がみられた。特に結合活性の強いハイブリドーマについて、限界希釀法によるクローニングに供した。クローン化したハイブリドーマの培養上清を同様にEIAのスクリーニングに供し、ヒト・フィブリン結合能の強いものを選択した。これらの結果、高濃度ヒト・フィブリノーゲン存在下でフィブリンに特異結合するMoAb産生マウスハイブリドーマFIB1-11およびFIB2-11が得られた。これらから得られたMoAb FIB1-11およびFIB2-11の免疫グロブリン・クラス、サブクラスはオーケターロニー法による測定で、いずれもIgG₁ (κ鎖) であった。

【0037】

【参考例9】マウス抗活性化血小板抗体産生ハイブリドーマの作製

①免疫

クエン酸採血で得たヒト新鮮血より遠心分離法で洗浄血小板を取得した。血小板約10⁹個にトロンビン0.1単位/mlを添加し、37°Cで5分間インキュベートしたのち、BALB/cマウスの腹腔内に (n=10; 約10⁹ケ/1ml/マウス) 注射した。2週毎に6-8回免疫した。

②細胞融合

参考例8-③に記載のマウス骨髄腫細胞P3U1の代わりにNS-1を用いて、以下全く同様の方法で細胞融合を実施した。

③ハイブリドーマの選択およびクローニング

血小板結合マイクロプレートを用いる参考例2に記載のEIAでハイブリドーマをスクリーニングし、以下参考例8-④と同じ要領で抗活性化血小板MoAb産生ハイブリドーマを取得した。これらの結果、ヒトおよびウサギ活性化血小板に特異的に結合するMoAb産生ハイブリドーマ2T60が得られた。抗体2T60の免疫グロブリン・クラス、サブクラスはオーケターロニー法による測定で、IgG₁ (κ鎖) であった。

【0038】

【参考例10】マウス抗tPAムテイン・モノクローナル抗体産生ハイブリドーマの作製

①免疫

tPA 200 μg/ml生理食塩水溶液に等量のフロント完全アジュバントを添加し十分乳濁後、BALB/cマウス(♀, 20 μg/0.2ml/マウス)に腹腔および背部皮下投与し、2~3週間隔で追加免疫を実施した。3回の追加免疫後、10日で最大の血清抗体価を示した個体について、tPA抗原液(50 μg/0.1ml生理食塩水/マウス)を静脈内投与した。

②細胞融合

参考例8-③に記載の方法に従い、細胞融合を実施した。

③ハイブリドーマの選択およびクローニング

tPAムテイン結合マイクロプレートを用いる参考例4

に記載のEIAでハイブリドーマをスクリーニングし、以下参考例8-④と同じ方法でtPAムテイン結合活性を有する抗tPA MoAb産生ハイブリドーマを取得した。これらの中、参考例7に記載のフィブリン溶解反応中和試験で中和能を示さず、かつtPAおよびtPAムテインに特異結合する抗tPA MoAbを産生するハイブリドーマとしてマウスハイブリドーマTPA1-41を得た。抗体TPA1-41の免疫グロブリン・クラス、サブクラスはオーケターロニー法による測定でIgG₂b (κ鎖) であった。

【0039】

【参考例11】ヒトtPAをコードする遺伝子を含むプラスミドの構築

(1) cDNA含有プラスミドの単離:

ヒト包皮由来初代培養細胞mRNAより合成したcDNAをpCDベクター(Okayamaら、モレキュラー・セル・バイオロジー(Molecular Cell Biology), 3,280 (1983) 参照)に組み込んで作成した大腸菌X1776を宿主としたcDNAライブラリーを大阪大学微生物病研究所の

岡山博士より分与を受けた。このcDNAライブラリーよりアルカリ法(Birnboim, H. C. & Doly, J., ヌクレオティック・アシッズ・リサーチ(Nucleic Acids Research), 1, 1513 (1979))でプラスミドDNAを抽出し、このDNAを大腸菌DH1に感染させ、約2×10⁶個のcloneよりなる大腸菌DH1を宿主としたcDNAライブラリーを作成した。

【0040】上記大腸菌DH1を用いたcDNAライブラリーをニトロセルロースフィルター(ミリポア社、HATFフィルター)上に約5×10⁴ clone/フィルターと

30 なるよう10枚まき、このフィルターをマスター・フィルターとしている各2枚ずつを1組としたレブリカフィルター計20枚を作成した。このレブリカフィルター上の大腸菌を0.5N NaOH溶液で溶かし、露出変性したプラスミドDNAをフィルター上に固定した(Grunstein, M. & Hogness, D. S., プロシージングスオブナショナルアカデミー オブサイエンス(Proc. Natl. Acad. Sci. USA) 72, 3961 (1975))。

【0041】一方、D. Pennicaらにより報告されている【ネイチャー(Nature) 301, 214 (1983)】ヒトtPA

40 のアミノ酸配列をもとにアミノ酸No.248-260(Asn-Arg-Arg-Leu-Thr-Trp-Glu-Tyr-Cys-Asp-Val-Pro-Ser) (配列番号3), アミノ酸No.489-501(Arg-Met-Thr-Leu-Val-Gly-Ile-Ile-Ser-Trp-Gly-Leu-Gly) (配列番号4) およびアミノ酸No.516-527-stop(Asn-Tyr-Leu-Asp-Trp-Ile-Arg-Asp-Asn-Met-Arg-Pro-Stop) (配列番号5) をもとにこれらのアミノ酸配列に対応する塩基配列を有するオリゴヌクレオチド各々 AAC CCC ACC CTG ACG TGG GAG TAC TGT GATGTG CCC TCC (配列番号6); CG C ATG ACT TTG GTG GGC ATC ATC ACC TCG GGCCTG CGC (配列番号7); AAC TAC CTA GAC TGG ATT CGT GAC AA

C ATG CGA CGGTGA (配列番号8) を化学合成した。このオリゴヌクレオチドに対してT4ポリヌクレオチドキナーゼ (宝酒造製) を用いて50μlの反応液 [オリゴヌクレオチド0.1μg, 50mM Tris-HCl pH8.0, 10mM MgCl₂, 10mMメルカプトエタノール, 50μCi γ -³²P-ATP (>5000Ci/mmol), 3ユニット T4ポリヌクレオチドキナーゼ] 中で37°C 1時間反応させ、オリゴヌクレオチドの5'末端を³²Pで標識した。上記方法で標識したオリゴヌクレオチド二種をプローブとして、別々に、DNAを固定したレブリカフィルターに会合させた。会合反応は10μCiのプローブを含む5×SSPE [180mM NaCl, 10mM リン酸ナトリウム緩衝液 (pH 7.4), 1mM EDTA], 5×Denhardt's, 0.1% SDS, 100μg/mI変性サケ精子DNA溶液10mI中で、35°C 16時間行い、反応後フィルターを5×SSC [0.15M NaCl, 0.015M クエン酸ナトリウム緩衝液] 0.1% SDS溶液で室温で30分ずつ3回さらに45°C 30分ずつ2回洗浄した [T. Maniatisら、モレキュラー・クローニング (Molecular Cloning) Cold Spring Harbor Laboratory, p. 309(1982)]。

【0042】洗浄したフィルターよりラジオオートグラムをとり、三種類のプローブの全部に対して反応する菌株を一組2枚のレブリカフィルターのラジオオートグラムを重ね合わせることにより探した。この方法により5×10⁵個のコロニーより三種類のプローブに対して反応する1株 [Escherichia coli K12 DH1/TPA13]を得た。この菌株よりプラスミドDNA (pTPA13)をアルカリ法 [ヌクレイック アシックス リサーチ (Nucleic Acids Research), 1, 1513(1979)]によって抽出精製し、cDNAの長さを検討したところ2.3kbであることが明らかになった。しかし、このcDNA鎖長ではN末端側までコードしていないことがわかった。

【0043】(2)ヒトTPA cDNAを含むファージの単離
 λ gt10をベクターとしたヒトニューロblastoma、cDNAライブラリー (クローニングテック社)を大腸菌C600、Hf1Aを宿主として、軟寒天ブレート上に、約1×10⁵クローンずつ、10枚まき、これを、ニトロセルロースフィルター (ミリポア社、HATFフィルター) 上に移した後、0.5N NaOH溶液でとかし露出変性したファージDNAをフィルター上に乾燥固定した。 [Maniatisら、「モレキュラー・クローニング (Molecular Cloning)」Cold Spring Harbor Laboratory, p320, 1982]。一方参考例11の(1)で得られたプラスミドpTPA13を制限酵素EcoRIおよびNarIで切断して得られる0.3KbのDNA断片をニックトランスレーション法 (Maniatisら、同上p109)により³²P標識し、プローブとした。標識したプローブと、DNAを固定したフィルターを、標識プローブを含む、5×SSPE (0.9M NaCl 50mM リン酸ナトリウム緩衝液 (pH7.4),

5mM EDTA), 50%ホルムアミド、5×Denhardt's, 0.1% SDS, 100μg/mI変性サケ精子DNA溶液10mI中で42°C、16時間、会合反応を行い、反応後フィルターを2×SSC (1×SSC=0.15M NaCl, 0.015Mクエン酸ナトリウム), 0.1% SDS溶液中で室温で30分ずつ2回、1×SSC, 0.1% SDS溶液中で68°Cで30分ずつ2回洗浄した。洗浄したフィルターを乾燥させた後、ラジオオートグラムをとり、プローブと反応するクローニングを検索した。この方法により得られたクローニングTPA7よりDavisらの方法 [Davisら、「アドバンスト・バクテリアル・ジェネティクス (Advanced Bacterial Genetics)」, Cold Spring Harbor Laboratory (1980)]によりファージDNAを抽出し、数種の制限酵素を用いて検討した結果、クローニングTPA7はpTPA13で欠けているヒトTPAのN末端側をコードするcDNAを有することがわかった。以上の結果、得られたpTPA13および λ TPA7のcDNA部分を組み合わせることにより、ヒトTPAのコード領域全体をカバーすることができた。また得られたcDNA部分の塩基配列をジテオキシヌクレオチド合成鎖停止法 [Messingら、「ヌクレイック・アシックス・リサーチ (Nucleic Acids Research)」9, 309, (1981)]によって決定した。報告されている塩基配列と比較した結果、アミノ酸No.129(Arg)をコードする塩基配列 (No.552-554) CGGがTGGにかわっており、コードされるアミノ酸がArg→Trpに置き換っていることがわかった (図3) (配列番号9)。

【0044】(3)動物細胞発現用プラスミドの構築
 上記した λ TPA7をEcoRIで切断し、0.8kbのDNA断片を得た。一方プラスミドpTB652 [サイエンス (Science) 236: 1116-1120, (1987)]をEcoRIで切断して得たアンビシリントリニティ遺伝子、MuLV-LTRおよびSV40プロモーターを含む断片 (約4.2kb)を調製し、両者をligationしてプラスミドp λ TPA7E0.8を構築した。このp λ TPA7E0.8をNarIおよびC1aIで消化して得た2.1kbのDNA断片とプラスミドpTPA13をNarIおよびC1aIで消化して調製したTPA cDNAを含む断片をligateし、プラスミドpTB920を構築した (図4)。次にpTB920をBamHIで消化して得た2.7kbのDNA断片を、pTB399 [セルストラクチャー アンド ファンクション (Cell Structure and Function) 12: 208-217, 1987]のBamHI部位に挿入してpTB926を構築した。このプラスミドをC1aIで消化して4.0kbの断片を調製し、pTB348 (Cell Structure and Function 12: 205-217, 1987)のC1aI部位に組み込んでプラスミドpTB927を構築した (図5)。

【0045】
 【参考例12】ヒトTPA cDNAの動物細胞での発現
 サルCOS-7細胞 [「セル (Cell)」, 27, 279-288 (198

1)】を5%胎児牛血清を含むD M E M培地で単層培養(ファルコン径60mmプラスチックディッシュ)した後、同培地で培地交換した。交換の4時間後に公知の方法(Grahamら、「ヴィロロジー(Virology)」, 52, 456(1973))に従いディッシュ1枚当たりプラスミドp T B 920またはp T B 927のDNA 10 μ gを含むカルシウムホスフェートゲルを調製し細胞に添加し、p T B 920感染細胞またはp T B 927感染細胞をそれぞれ得た。さらにその4時間後グリセロール処理して5%胎児牛血清を含む培地で上記p T B 920感染C O S - 7細胞またはT B 927感染C O S - 7細胞の培養を続けた。70~72時間後に培養上清を集め、上清中のt P A量を参考例3に記載のE I A法により測定したところ200 μ g~400 μ g/m lのt P Aが検出され、上記c DNAが正しくヒトt P Aをコードしていることが認められた。

【0046】

【参考例13】 ムテインをコードする塩基配列を有する組換えDNAの製造
参考例11に記載のプラスミドp T B 927をBgl IIで消化して2.1kbのDNA断片を得、これをさらにApaL IおよびBstY Iで消化して1.3kbの断片を得た。このDNAの両端をKlenowフラグメントを用いた反応により平滑化した後、プラスミドpUC118のSma I部位に組み込みプラスミドp T B 1127を構築し、合成オリゴヌクレオチドを使用する特定部位指向性変異誘発の鉄型として用いた。特定部位指向性変異はOligonucleotide-directed in vitro Mutagenesis System(アマシャム社)と合成オリゴマーを用いて行った。用いた合成オリゴマーの配列を下に示す。

5'-ACTGTTCCCTCAGACATATGACCGGTCTCCAGAA-3'

(36mer) (配列番号10)

この合成オリゴマーを用いることにより、C¹⁷³はMに置換されると同時にNde Iの認識部位が導入された、ヒトt P A c DNAを構築することができた(図6)。即ち、t P Aのアミノ酸番号1~173までのF E K 1ドメインの欠失したt P Aムテインが得られた。このようにして得られたt P Aムテイン、t P A-1の塩基配列およびそれから推測されるアミノ酸配列を図7(配列番号11)に示す。このムテインとt P Aとの違いを示すと以下のようである。

アミノ酸番号	174	295	303	527
t P AACSE.....	IFAKHRRSPGERFL.....	P	
t P A-1MSE.....	IFAKHRRSPGERFL.....	P	

上記のようにして得られたプラスミドをp T B 1128とした(図8)。

【0047】

【参考例14】 ヒトt P Aのムテインをコードする遺伝子による大腸菌の形質転換

(1) ヒトt P Aのムテイン発現用プラスミドp T B 11

33の構築

前記参考例13で得られたp T B 1128をNde IおよびBam H Iで切断し、各々約1.1kbのDNA断片を得た。この断片をプラスミドp E T 3 c (Studier, F. W. (Brookhaven National Labs. U.S.A.)より分与をうけた)のNde I-Bam H I部位に挿入して、プラスミドp T B 1133を構築した(図8)。

(2) 形質転換

次に大腸菌MM294株に、T 7ファージのRNAポリメラーゼ遺伝子を組み込んだファージDE3 (Studier, F. W. ら、ジャーナル オブ モレキュラー バイオロジー (J. Mol. Biol.), 189: 113-130 (1986))を溶原化させ、さらにT 7ファージのリゾチーム遺伝子をもつプラスミドpLysS (Studier, F. W. ら、ジャーナル オブ モレキュラー バイオロジー (J. Mol. Biol.), 189: 113-130 (1986))を導入し、大腸菌MM294(DE3)/pLysS株を作製した。この大腸菌株にp T B 1133を導入し、大腸菌MM294(DE3)/pLysS, p T B 1133をつくった。この菌株はヒトt P Aムテインt P A-1を産出するように作製されていた。

【0048】

【参考例15】 t P A-1発現大腸菌の培養

参考例14で得た大腸菌MM294(DE3)/pLysS, p T B 1133を、200m l容技付き三角フラスコ内の1%バクトリブトン(ディフコラボラトリーズ、アメリカ)、0.5%バクトイーストエキス(ディフコラボラトリーズ、アメリカ)、0.5%NaCl、100 μ g/m lアンビシリソナトリウムおよび10 μ g/m lクロラムフェニコールを含む液体培地40m lに接種して37°Cにて一晩振盪培養した。

30 この培養液10m lを1,000m l容三角フラスコ内の1%バクトリブトン、0.5%バクトイーストエキス、0.5%NaCl、100 μ g/m lアンビシリソナトリウムおよび10 μ g/m lクロラムフェニコールを含む上記液体培地200m lに添加して37°Cにて4時間、ついでイソプロピル-β-D-チオガラクトビラノシド(和光純薬工業株式会社)0.1m Mを添加してさらに4時間振盪培養して培養液を得た。この培養液を遠心分離し、菌体を集め-80°Cで凍結して保存した。

【0049】

40 【参考例16】 t P A-1蛋白質の抽出、賦活化および精製

①抽出、賦活化

参考例15で得たt P A-1発現大腸菌凍結菌体を50m Mトリス塩酸緩衝液(pH 8.0)50m lに懸濁した。懸濁液を超音波処理(2A×2分間、2回)にかけ、溶菌液を得た後18,900×gで20分間遠心分離して沈殿物を得た。この沈殿物を10m Mトリス塩酸緩衝液(pH 8.0)50m lで洗浄して、18,900×gで20分間遠心分離して沈殿物を得た。さらにこの沈殿物を0.25% (v/v)トライトニンX-100を含む10m Mトリス塩酸緩衝液(pH 8.0)で洗浄

して、 $18,900 \times g$ で20分間遠心分離して沈殿物を得た。この沈殿物に抽出用緩衝液(7M塩酸グアニジン-0.1M KH₂PO₄(pH7.5)-5mM 2-メルカプトエタノール)25mLを加えて4°Cにて一晩攪拌した。この抽出液を $18,900 \times g$ で20分間遠心分離して抽出上清25mLを得た。抽出上清25mLを7M塩酸グアニジン-0.1M リン酸カリウム緩衝液(pH7.5)-5mM 2-メルカプトエタノールに対して透析した後、脱気した賦活化用緩衝液[2.5M尿素-50mMトリス塩酸緩衝液(pH8.75)-10mM NaCl-5mM EDTA-10mMリシン-0.5mM還元型グルタチオン-0.3mM酸化型グルタチオン]1,250mLに加えて攪拌した後、15°Cにおいて保温した。

【0050】の精製

賦活化されたtPA-1溶液の各々1,250mLを50mMトリス塩酸緩衝液(pH8.0)-0.01%Tween80に対して透析した。透析内液を $18,900 \times g$ で20分間遠心分離して透析上清を得た。この透析上清を50mMトリス塩酸緩衝液(pH8.0)で平衡化したQAEトヨバール550C(東ソー社)カラム(カラム容量70mL)に負荷して蛋白質を吸着させ、次に同緩衝液でカラムを洗浄した後0~1.0M NaClの直線濃度勾配溶出法(溶出緩衝液容量600mL)にてtPA-1を溶出した。活性画分を0.5M NaCl-50mMトリス塩酸緩衝液(pH8.0)-0.01%Tween80で平衡化したベンザミンセファロース6B(ファルマシア社、スウェーデン)カラム(カラム容量20mL)に負荷して活性型tPA-1を吸着させ、次に同緩衝液でカラムを洗浄した後0.2Mアルギニン-0.5M NaCl-50mMトリス塩酸緩衝液(pH8.0)-0.01%Tween80で活性型tPA-1を溶出した。以上の精製操作により、SDS-PAGEで単一バンドを示すtPA-1標品を得た。すなわち、分子量は還元条件下で39,500、非還元条件下で35,000であった。

【0051】

【参考例17】 ムテイン(tPA-6')をコードする塩基配列を有する組換えDNAの製造

参考例13に記載のプラスミドpTB1128を合成オリゴヌクレオチドを使用する特定部位指向性変異誘発の鉄型として用いた。特定部位指向性変異はOligonucleotide-directed *in vitro* Mutagenesis System(アマシャム社)と合成オリゴマーを用いて行った。用いた合成オリゴマーの配列は下に示すとおりである。

5'-GGGCGACTCTTCG TGCTTGGCAA
A-3'

(24mer)(配列番号12)

この合成オリゴマーを用いることにより、R²⁹⁸⁻²⁹⁹はE²⁹⁸⁻²⁹⁹に置換されると同時にMb₀IIの認識部位が導入されたFEK1ドメイン欠失ヒトtPA cDNAを構築することができた(図9)。この合成オリゴマーを用いて得られたtPAムテイン、tPA-6'の塩基配

列およびそれから推測されるアミノ酸配列を図10(配列番号13)に示す。このムテインとtPAとの違いを示すと以下のようである。

アミノ酸番号	174	295	303	527
tPAACSE.....	IPAKHRRSPGERPL.....P		
tPA-6'MSE.....	IPAKHEESPGERPL.....P		

この合成オリゴマーを用いた結果得られたプラスミドをpTB1038とした。

【0052】

【参考例18】 ヒトtPAのムテイン(tPA-6')をコードする遺伝子による大腸菌の形質転換

(1) ヒトtPAのムテイン発現用プラスミドpTB1277の構築

前記参考例17で得られたpTB1038をNdeIおよびBamHIで切断し、約1.1kbのDNA断片を得た。この断片をプラスミドpET3c(Studier, F. W. (Brookhaven National Labs. U.S.A.)より分与をうけた)のNdeI-BamHI部位に挿入して、プラスミドpTB1277を構築した(図9)。

(2) 形質転換

次に参考例14で示した大腸菌MR94(DE3)/pLysS株にpTB1277を導入し、大腸菌MR94(DE3)/pLysS, pTB1277をつくった。これらの菌株はヒトtPAムテインtPA-6'を産出するように作製されていた。

【0053】

【参考例19】 tPA-6'蛋白質の抽出、賦活化および精製

①抽出

参考例18で得た大腸菌MR94(DE3)/pLysS, pTB1277を参考例15で示した培養条件下に培養し、tPA-6'発現大腸菌体を得た。このtPA-6'発現大腸菌菌体20gを50mMトリス塩酸緩衝液(pH8.0)0.2リットルに懸濁した。懸濁液を超音波処理(2A×2分間、2回)にかけ、次いで $18,900 \times g$ で20分間遠心分離して沈殿物を得た。この沈殿物を0.5% (v/v)トライトンX-100を含む50mMトリス塩酸緩衝液(pH8.0)0.2リットルで洗浄して、 $18,900 \times g$ で20分間遠心分離して沈殿物を得た。さらにこの沈殿物を50mMトリス塩酸緩衝液(pH8.0)0.2リットルで洗浄して、 $18,900 \times g$ で20分間遠心分離して沈殿物を得た。この沈殿物に抽出用緩衝液(7M塩酸グアニジン-0.1M KH₂PO₄(pH7.5)-0.1M 2-メルカプトエタノール)0.1リットルを加えて4°Cで一晩攪拌した。この抽出液を $18,900 \times g$ で20分間遠心分離して抽出上清0.1リットルを得た。

②賦活化

賦活化用緩衝液[2.5M尿素-50mMトリス塩酸緩衝液(pH8.75)-10mM NaCl-5mM EDTA-10mMリシン-0.5mM還元型グルタチオン]20リットルに、抽出上清0.1リットルを加えて15°Cで1時間攪拌した後、酸化型グルタチオンを

最終濃度0.3mMになるように加えて15°Cで保温した。

③精製

賦活化されたtPA-6'溶液を限外濾過法により2リットルに濃縮し、次に50mMトリス塩酸緩衝液(pH8.5)0.01%Tween80に対して透析した後、18,900×gで20分間遠心分離して透析上清2リットルを得た。この透析上清を50mMトリス塩酸緩衝液(pH8.5)で平衡化したQAEトヨバール550C(東ソー社)カラム(カラム容量150ml)に負荷して蛋白質を吸着させ、次に同一緩衝液0.3リットルでカラムを洗浄した後0~1.0MNaClの直線勾配溶出法(溶出緩衝液容量1,200ml)を用いてtPA-6'を溶出した。活性画分0.2リットルを集め、50mMトリス塩酸緩衝液(pH8.5)-0.01%Tween80に対して透析した。この透析内液0.2リットルを同一緩衝液で平衡化したリシンセファロース4B(ファルマシア社、スウェーデン)カラム(カラム容量40ml)に負荷してtPA-6'を吸着させた。0.5MNaCl-50mMトリス塩酸緩衝液(pH8.5)-0.01%Tween80 80mlでカラムを洗浄した後、0.2Mアルギニン-0.5MNaCl-50mMトリス塩酸緩衝液(pH8.5)-0.01%Tween80でtPA-6'を溶出した。以上の精製操作により、SDS-PAGEで單一バンドを示すtPA-6'標品38mgを得た。すなわち、分子量は還元条件下で39,000であり、非還元条件下では33,000であった。この値は一次構造より推定される理論値(tPA-6'=39,561)によく一致した。

【0054】

【参考例20】抗活性化血小板-tPAマテイン・ハイブリッド抗体測定用EIA
参考例2で作製した抗活性化血小板感作プレートに被検ハイブリドーマ培養上清を添加し、室温で2時間反応させた。次いでPBS-Twで洗浄後ビオチン標識したtPAを添加し、さらに室温で2時間反応させた。次ぎにアビジン-HRP複合体を添加し室温で1時間反応後、固相に結合したHRP活性を参考例1に示した方法で測定した。

【0055】

【実施例1】マウス抗ヒトフィブリノーゲン特異抗体産生ハイブリドーマの作製

①免疫原および寛容原の調製

公知の固相合成法によりペプチド合成機(アプライド・システム社、モデル430A型)を用いて次式に示すA、B2種のペプチドを合成した。

A : H-Gly-His-Arg-Pro-Leu-Asp-Lys-Cys-OH
(8-merr, 配列番号14)

B : H-Phe-Phe-Ser-Ala-Arg-Gly-His-Arg-Pro-Leu-Asp-Lys-OH
(12-merr, 配列番号15)

ペプチドAはヒトフィブリノーゲンβ鎖N末端ペプチド(1-7)-Cysに相当し、ペプチドBは上記フィブリノーゲンβ

鎖N末端ペプチド(1-7)をC端側に含む12個のアミノ酸からなるフィブリノーゲンβ鎖部分ペプチドである。予めメターマレイミドベンゾイル-N-ヒドロキシ・スクシンイミドエステル(以下、MBSと略記することがある)を用いてマレイミド化したキーホール・リンペット・ヘモシアニン(以下、KLHと略記することがある)にペプチドAを添加し、KLH 1モル当たりペプチドA 7分子が結合した(ペプチドA),-KLH複合体を調製した。ペプチドBはMBSでマレイミド化したのち、一方でS-アセチル・メルカブト・無水コハク酸とヒドロキシル・アミンとで連続処理することによりスルフヒドリル基を導入したD-GL[D-グルタミン酸とD-リジンとの共重合体:分子量34,300, D-Glu:D-Lys=60:40(ピアース・ケミカルズ社製)]に添加し、D-GL 1分子当たりペプチドB 4分子が結合した(ペプチドB),-D-GL複合体を調製した。

【0056】②免疫法

免疫原として(ペプチドA),-KLH複合体を、寛容原として(ペプチドB),-D-GL複合体を用いる免疫法を採用した。まず初回免疫5日前および3日前にBALB/cマウス(♀; n=10)に寛容原1mg/生理食塩水溶液400μl/マウスを腹腔内投与した。次いで免疫原の生理食塩水溶液に等量のフロイント不完全アジュバントを添加し十分乳濁後、40μg/0.2ml/マウスを腹腔および背部皮下投与した。2~4回目の追加免疫は、いずれも免疫原をフロイント不完全アジュバントに乳濁し初回と同量を皮下投与したが、それぞれの追加免疫5日前および3日前には必ず寛容原500μg/生理食塩水溶液200μl/マウスを腹腔内投与した。4回免疫後10日で最大の血清抗体価を示した個体について、免疫原(50μg/0.2ml/マウス)を静脈内投与した。

③細胞融合

参考例8-③に記載の方法に従い、細胞融合を実施した。

④ハイブリドーマの選択およびクローニング

フィブリノーゲンおよびフィブリノーゲンモノマー結合プレートを用いる参考例5および1に記載のEIAでハイブリドーマをスクリーニングし、以下、参考例8-④と同じ要領で抗ヒトフィブリノーゲン特異抗体産生ハイブリドーマFTB2-133を取得した。得られた結果は図11に示した通りであった。該図においてはフィブリノーゲンに対する反応性(○:参考例5に記載のEIA)およびフィブリノーゲンに対する反応性(●:参考例1に記載のEIA)を示している。

【0057】抗ヒトフィブリノーゲン特異抗体産生ハイブリドーマFTB2-133はフィブリノーゲンにのみ結合能を示し、フィブリノーゲンにはほとんど反応しなかった。FTB2-133抗体の免疫グロブリンクラス、サブクラ

スはオーケターロニー法による測定で IgG₁ (κ鎖) であった。

【0058】⑤HAT感受性株の取得

④で得られたHAT抵抗性のFTB2-133細胞株を、まず1μM濃度の8-AZG添加培地で培養し、次いで順次8-AZG濃度を2~5倍づつ上昇させ、培養を続けた。100μMの8-AZGに耐性となった細胞株についてHAT感受性および抗ヒトフィブリン特異抗体産生性を測定し、目的の細胞株を選定した。得られた細胞株は元のHAT抵抗性株と同等の抗体産生性を有し、かつHAT添加培地で100%死滅した。

【0059】

【実施例2】マウス抗フィブリン-抗tPAムテインb_sMoAbの製造(1)

①免疫

参考例16で作製したtPA-1 100μg/ml生理食塩水溶液を用いて、参考例8-②と同じ要領でマウスを免疫した。

②細胞融合

* 最終免疫後3日で脾臓を摘出し、脾臓細胞懸濁液を常法により調製した(約10⁸個)。次いで参考例1-⑤で取得したHAT感受性の抗ヒトフィブリン抗体産生ハイブリドーマFTB2-133 2×10⁷個を添加し、参考例8-③に示した要領で細胞融合およびHAT選択を実施した。

③ハイブリッド・ハイブリドーマの選択およびクローニング tPA結合マイクロプレートを用いる参考例3に記載のEIAおよび抗フィブリン-抗tPAムテインb_sMoAb活性を測定する参考例6に記載のEIAを用いてハイブリッド・ハイブリドーマをスクリーニングし、以下参考例8-④と同じ方法により抗フィブリン-抗tPAムテインb_sMoAb産生トリオーマTAF 1-42, TAF 1-79およびTAF 1-228を取得した。それぞれのトリオーマ培養上清をEIAに供した結果を表1に示す。

【0060】

【表1】

吸光度(492nm)

トリオーマ	吸光度(492nm)	
	抗tPA抗体活性 ¹	二重特異性抗体活性 ²
TAF 1-42	1. 476	1. 467
TAF 1-79	1. 475	1. 419
TAF 1-228	1. 451	0. 658
コントロール ³	0. 051	0. 199

1) 参考例3参照。

2) 参考例6参照。

3) FIB 1-11抗体産生ハイブリドーマ(参考例8-④参照)の培養上清を使用。

3種のb_sMoAb TAF 1-42, 1-79および1-228はオーケターロニー法による測定の結果いずれも IgG₁ (κ鎖) であった。

【0061】④ハイブリッド抗体の精製

予め0.5ml鉱油を腹腔内投与したBALB/cマウス1群5匹に5×10⁶個/マウスのb_sMoAb産生トリオーマTAF 1-42, TAF 1-79およびTAF 1-228をそれぞれ腹腔内接種した。約10~15日後に腹水の貯留が見られた。抗体の精製は常法により、45~50%飽和硫酸アンモニウムで分画後、参考例8-①に記載のヒト・フィブリンβ鎖N末端ペプチド(1-11)-Cysを結合したセルロファイン・カラムに供し、アフィニティークロマトグラフィーで抗フィブリン抗体活性を有するIgG画分を取得した。次いでヒドロキシ・アバタイト・カラムを用いる高速液体クロマトグラフィーによりb_sMoAbを分離、精製した。こうして腹水各5mlからb_sMoAb TAF 1-42, TAF 1-79およびTAF 1-228がそれぞれ12mg, 4mg, 30mg得られた。得られた結果を図12に示す。矢印で示したピークが、参考例6に記載のEIAでb_sMoAb活性陽性を示したIgG画分

であった。

【0062】

【実施例3】マウス抗フィブリン-抗tPAムテインb_sMoAbの製造(2)①細胞融合

参考例8-④で取得した抗ヒトフィブリン抗体産生ハイブリドーマFIB 1-11および参考例10-③で取得した抗tPA抗体産生ハイブリドーマTPA 1-41を、それぞれ0.5μg/ml FITCおよび1.5μg/ml TRITC含有イスコフーハムF-12混合培地で37°C、30分間インキュベートし、蛍光染色した。次いで、LSM溶液(和光純薬工業K.K.販売)を添加し死細胞を除去したのち、両ハイブリドーマを1:1の割合で混じ、PEG 6000を用いて参考例8-③に記載の方法で細胞融合した。37°Cで2時間インキュベート後、FACSに供することによりフルオレセインおよびローダミン二重染色された細胞25,000個を分取し、次にフィーダーとしてマウス胸腺細胞を5×10³個/ウェル播種した96穴マイクロプレートに、上記の二重染色細胞を10個/ウェルの割合で播種し培養した。

②ハイブリッド・ハイブリドーマの選択およびクローニング

融合後1~2週で細胞増殖のみられたウェルの培養上清を、それぞれ参考例1, 3および6に記載のEIAに供し抗体活性を測定した。高いハイブリッド抗体活性を示

したウエルについて限界希釈法によるクローニングを実施し、目的の二重特異性抗体産生マウス・ハイブリッド・ハイブリドーマ（テトラオーマ）FT2-14を取得した。

【0063】③ハイブリッド抗体の精製

予め0.5ml鉛油を腹腔内投与したBALB/cマウス6匹に 5×10^6 個/マウスのマウスハイブリッド・ハイブリドーマFT2-14を腹腔内接種した。約10～20日後に腹水の貯留が認められたのでそれを採取し、45～50%飽和硫酸アンモニウムで塩析して1gG画分を得た。次いで20mM PBSで平衡化したフィブリン結合セルロファイン・カラムに供し、0.2Mグリシン・塩酸緩衝液（pH 2.9）で溶出した。得られた蛋白画分を1N NaOHで中和後、10mMリン酸カリウム緩衝液（pH 6.8）で透析し、同じ緩衝液で平衡化したヒドロキシアバタイト・カラムを用いる高速液体クロマトグラフィーに供した。リン酸カリウム緩衝液（pH 6.8）10mMから210mMの塩濃度勾配溶出法を用いて二重特異性ハイブリッド抗体FT2-14を精製した。約50mlの腹水液から47mgの精製抗体FT2-14を得た。得られた精製bsMoAb FT2-14を、参考例6に記載の抗フィブリン-抗tPAムテイン・ハイブリッド抗体測定用EIAの供した結果を図14に示す。ヒトフィブリンおよびtPA-6'の相方に強い結合活性を有することが明らかとなった。

【0064】④F (a b')₂の精製

得られた精製抗体FT2-14を20mM酢酸緩衝液（pH 3.5）に溶解し、ペプシン不溶化カラム（5mgペプシン/2.5mlセルロファイン・ゲル）に供した。37°Cにおいて3ml/時間の流速でゆっくりと溶出し、ペプシン消化物を得た。1N NaOHでpH 7.5に調整後プロテインA・カラムに供し、PBS（pH 7.5）で溶出する蛋白画分を分取した。さらにフィブリン結合カラムに供し、PBSでカラムを溶出・洗浄後、pH 2.5のグリシン-塩酸緩衝液でフィブリン結合活性を有するF (a b')₂画分を溶離した。約4.0mgのFT2-14（全1gG分子）から4.3mgのFT2-14 F (a b')₂画分を取得した。

⑤二重特異性抗体活性

⑥で得られたF (a b')₂画分を参考例6に記載のEIAに供し二重特異性抗体活性を測定した。得られた結果は図14に示した通りであった。図中、抗フィブリン・抗tPAムテインbsMoAb FT2-14（●）およびそのF (a b')₂画分（○）に関する結果を示す。FT2-14 F (a b')₂画分は1gG全分子と同様にフィブリンおよびtPAムテインの両者に強い結合能を示した。

【0065】

【実施例4】免疫複合体液の調製

一定量のtPA（最終濃度0.1μg/ml）および参

考例19に記載のtPA-6'（最終濃度0.5μg/ml）に種々の濃度の実施例2で得られたマウス抗フィブリン-抗tPAムテインbsMoAbを添加し、室温で20分間免疫反応させて免疫複合体液を調製した。

【0066】

【実施例5】フィブリン溶解能の増強効果

公知の方法[D. Collenら: スロンボーシス・アンド・ヘモスタシス (Thromb. Haemostasis), 45, 225 (1981)]に従い、血漿凝塊溶解試験（plasma clot lysis assay）を実施した。すなわち、実施例4で得られた免疫複合体にヒト血漿を添加し、次いでヒト・トロンビンを最終濃度1.0単位/mlとなるように加えて凝固させた。溶解度分析計（Euglobulin lysis analyzer "ELT-6" MebanixCo.）を用いて血漿の濁度を経時的に観察し、溶解に要する時間を測定した。非修飾型tPAに対しては3種のbsMoAb（TAF 1-42, TAF 1-79およびTAF 1-228）はいずれもフィブリン溶解能の増強効果を示さなかった。tPA-6'に対しては2種のbsMoAb（TAF 1-42およびTAF 1-79）が増強効果を示さなかったが、TAF 1-228は図13に示したようにtPA-6'の線溶活性として用量依存的に最大5倍以上の増強効果を示した。図中、縦軸は非修飾tPA単剤（●）もしくはtPAムテインtPA-6'単剤（○）の溶解能を100%とした時の、それぞれtPA/抗体複合体（●）もしくはtPA-6'/抗体複合体（○）の溶解能を表す。

【0067】

【実施例6】マウス抗活性化血小板-tPAムテイン・bsMoAbの製造

①単特異性抗体の精製

参考例9および10にそれぞれ記載の抗活性化血小板抗体産生ハイブリドーマ2T60および抗tPAムテイン抗体産生ハイブリドーマTPA1-41を用いて、実施例2-④に記載の方法に従い腹水化し、さらに精製して1gG抗体画分を得た。

②F (a b')₂の調製

上記①で調製した2種の抗体1gG画分をそれぞれ公知の方法[M. Marianiら: モレキュラー・イムノロジー (Mol. Immunol.), 28, 69 (1991)]に供し、フィシン（シグマ社販売）を用いてトリス・塩酸緩衝液中（pH 7.0）で分解した。L-システイン（和光純薬販売）を添加し（最終濃度1mM）反応を活性化したのち、37°Cで4時間インキュベートした。100mM N-エチルマレイミドの添加で反応停止後、得られた抗体フラグメント溶液をPBS（pH 7.4）で透析した。それぞれの抗体溶液を同じPBSで平衡化したプロテインAカラム（2.6×4.7cm）に供し、素通り画分を分取した。さらに、TSKゲル（東洋ソーダ製造）を用いるゲル濾過カラム・クロマトグラフィーに供してF

(a b')₂ 画分を調製した。

③ヘテロダイメリックF (a b')₂の調製

上記②で調製した2T60抗体由来のF (a b')₂をDTTで還元後、o-フェニレンジマレイミドを用いる公知の方法 [M.J.Glennieら:ジャーナル・オブ・イムノロジー (J.Immunol.), 139, 2367 (1987)] に従って2T60 F ab' 画分のスルフヒドリル基をマレイミド化した。一方上記②で調製したTPA1-41抗体由来のF (a b')₂を同様にDTTで還元後、セファデックスG25ゲル通過カラム・クロマトグラフィーを用いて過剰のDTTを分離ないし除去し、上記のマレイミド化2T60 F ab' にモル比1:1で添加し、チオエーテル結合により2種の異なった特異性を有する抗体F ab' をヘテロダイメリックに結合させた。得られる反応混合物をウルトロ・ゲルA c A 44カラム (LK B社販売) を用いるゲル通過カラム・クロマトグラフィーに供し、ヘテロダイメリックF (a b')₂を精製分取した。両单特異性抗体精製 Ig G標品10mgより目的の二重特異性F (a b')₂を抗体約11mgを得た。

④二重特異性抗体活性

③で調製したヘテロダイメリックF (a b')₂、画分を参考例20に記載のEIAに供し抗活性化血小板-抗tPAムテイン二重特異性抗体活性を測定した。得られた結果は図15に示した通りであった。

【0068】

10

【配列表】

配列番号: 1

配列の長さ: 12

配列の型: アミノ酸

トポロジー: 直鎖状

配列の種類: ベブチド

フラグメント型: N末端フラグメント

配列

Gly His Arg Pro Leu Asp Lys Lys Arg Glu Glu Cys

1 5 10 15.

20 【0071】配列番号: 2

配列の長さ: 16

配列の型: アミノ酸

トポロジー: 直鎖状

配列の種類: ベブチド

フラグメント型: 中間部フラグメント

配列

Phe Phe Ser Ala Arg Gly His Arg Pro Leu Asp Lys Lys Arg Glu Glu

1 5 10 15.

【0072】配列番号: 3

配列の長さ: 13

配列の型: アミノ酸

配列

Asn Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser

1 5 10.

【0073】配列番号: 4

配列の長さ: 13

配列の型: アミノ酸

配列

Arg-Met-Thr-Leu-Val-Gly-Ile-Ile-Ser-Trp-Gly-Leu-Gly

1 5 10.

【0074】配列番号: 5

配列の長さ: 12

配列の型: アミノ酸

トポロジー: 直線状

配列の種類: ベブチド

フラグメント型: C末端フラグメント-stop

配列

32

* 【発明の効果】本発明のハイブリッドbsMoAbは、tPAムテインの血栓溶解能を損なうことなく該tPAムテインと結合し、かつ血栓への親和性、選択性を大幅に増大することが可能である。従ってハイブリッドMoAbとtPAムテインとの1:1免疫複合体を作製することにより、選択的かつ効率的な血栓の溶解、除去が可能となる。

【0069】

【0070】

【配列表】

配列番号: 1

配列の長さ: 12

配列の型: アミノ酸

トポロジー: 直鎖状

配列の種類: ベブチド

フラグメント型: N末端フラグメント

配列

Gly His Arg Pro Leu Asp Lys Lys Arg Glu Glu Cys

1 5 10.

20 【0071】配列番号: 2

配列の長さ: 16

配列の型: アミノ酸

トポロジー: 直鎖状

配列の種類: ベブチド

フラグメント型: 中間部フラグメント

30* トポロジー: 直鎖状

配列の種類: ベブチド

* フラグメント型: 中間部フラグメント

★ トポロジー: 直鎖状

配列の種類: ベブチド

★ フラグメント型: 中間部フラグメント

☆ 配列

Asn-Tyr-Leu-Asp-Trp-Ile-Arg-Asp-Asn-Met-Arg-Pro

1 5 10.

【0075】配列番号: 6

配列の長さ: 39

配列の型: 核酸

トポロジー: 直鎖状

配列の種類: 他の核酸 合成DNA

(18)

特開平5-304992

33

34

AAC CCC AGG CTG ACG TCG GAG TAC TGT GAT GTG CCC TCC

39.

【0076】配列番号: 7

* トポロジー: 直鎖状

配列の長さ: 39

配列の種類: 他の核酸 合成DNA

配列の型: 核酸

*

配列

CCC ATG ACT TTG GTG CCC ATC ATC ACC TCG CCC CTG CCC

39.

【0077】配列番号: 8

* トポロジー: 直鎖状

配列の長さ: 39

配列の種類: 他の核酸 合成DNA

配列の型: 核酸

*

配列

AAC TAC CTA GAC TCG ATT CGT GAC AAC ATG CGA CCG TGA

39.

【0078】配列番号: 9

★起源

配列の長さ: 1800

ヒト包皮由来初代培養細胞

配列の型: 核酸

配列の特徴

鎖の数: 二本鎖

存在位置: 552..554

トポロジー: 直鎖状

特徴を決定した方法: E

配列の種類: cDNA to mRNA

★

配列

AAAACCTCTG CGAGGAAAGG GAAGGAGCAA GCC

GTGAATT TAAGGGACGC TGTGAAGCAA 60

TC ATG GAT GCA ATG AAG AGA CGG CTC TCC TGT GTG CTG CTG CTG TGT 107

Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu Leu Cys

-35 -30 -25

CGA GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT CCC CCA TTC AGA 155

Gly Ala Val Phe Val Ser Pro Ser Gln Glu Ile His Ala Arg Phe Arg

-20 -15 -10 -5

AGA GGA GCC AGA TCT TAC CAA GTG ATC TGC AGA GAT GAA AAA ACG CAG 203

Arg Gly Ala Arg Ser Tyr Gln Val Ile Cys Arg Asp Glu Lys Thr Gln

1 5 10

ATG ATA TAC CAG CAA CAT CAG TCA TGG CTG CCC CCT GTG CTC AGA AGC 251

Met Ile Tyr Gln Gln His Gln Ser Trp Leu Arg Pro Val Leu Arg Ser

15 20 25

AAC CCG GTG GAA TAT TCC TCG TCC AAC AGT CCC ACG GCA CAG TCC CAC 299

Asn Arg Val Glu Tyr Cys Trp Cys Asn Ser Gly Arg Ala Gln Cys His

30 35 40

TCA GTG CCT GTC AAA AGT TCC AGC GAG CCA AGG TGT TTC AAC CGG CGC 347

Ser Val Pro Val Lys Ser Cys Ser Glu Pro Arg Cys Phe Asn Gly Gly

45 50 55 60

ACC TCC CAG CAG GCC CTG TAC TTC TCA GAT TTC GTG TCC CAG TCC CCC 395

Thr Cys Gln Gln Ala Leu Tyr Phe Ser Asp Phe Val Cys Gln Cys Pro

65 70 75

GAA GGA TTT CCT CGG AAG TCC TGT GAA ATA CAT ACC ACG GCC ACG TCC 443

Glu Gly Phe Ala Gly Lys Cys Cys Glu Ile Asp Thr Arg Ala Thr Cys

80 85 90

TAC GAG GAC CAG CGC ATC ACC TAC AGC AGG CGC ACG TGG ACC ACA GCG GAG 491

Tyr Glu Asp Gln Gly Ile Ser Tyr Arg Gly Thr Trp Ser Thr Ala Glu

95 100 105

AGT GCC CCC GAG TCC ACC AAC TGG AAC AGC AGC GCG TTG GCC CAG AAG 539

Ser Gly Ala Glu Cys Thr Asn Trp Asn Ser Ser Ala Leu Ala Gln Lys

110 115 120

35 CCC TAC AGT GGG TCG AGG CCA GAC CCC ATC AGG CTG CCC CTG GGG AAC 587
 Pro Tyr Ser Gly Trp Arg Pro Asp Ala Ile Arg Leu Gly Leu Asn
 125 130 135 140
 CAC AAC TAC TCC AGA AAC CCA CAT CGA GAC TCA AAG CCC TGG TCC TAC 635
 His Asn Tyr Cys Arg Asn Pro Asp Arg Asp Ser Lys Pro Trp Cys Tyr
 145 150 155
 GTC TTT AAG GCG GGG AAG TAC AGC TCA GAG TTC TCC ACC ACC CCT CCC 683
 Val Phe Lys Ala Gly Lys Tyr Ser Ser Glu Phe Cys Ser Thr Pro Ala
 160 165 170
 TCC TCT GAG GGA AAC AGT GAC TCC TAC TTT CGG AAT CGG TCA GCC TAC 731
 Cys Ser Glu Gly Asn Ser Asp Cys Tyr Phe Gly Asn Gly Ser Ala Tyr
 175 180 185
 CGT CGC ACG CAC AGC CTC ACC GAG TCG GGT CCC TCC TGC CTC CCG TGG 779
 Arg Gly Thr His Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp
 190 195 200
 AAT TCC ATG ATC CTG ATA GCC AAG GTT TAC ACA GCA CAG AAC CCC AGT 827
 Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser
 205 210 215 220
 CCC CAG GCA CTG CGC CTG CGC AAA CAT AAT TAC TCC CGG AAT CCT GAT 875
 Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp
 225 230 235
 CGG GAT GCC AAG CCC TGG TCC CAC GTG CTG AAG AAC CGC AGG CTG ACG 923
 Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr
 240 245 250
 TGG GAG TAC TGT GAT GTG CCC TCC TGC TCC ACC TCC CCC CTG AGA CAG 971
 Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln
 255 260 265
 TAC ACC CAG CCT CAG TTT CCC ATC AAA CGA CGG CTC TTC CCC GAC ATC 1019
 Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile
 270 275 280
 CCC TCC CAC CCC TGG CAG CCT GCC ATC TTT CCC AAG CAC AGG AGG TCG 1067
 Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser
 285 290 295 300
 CCC GGA GAG CGG TTC CTG TCC CGG CGC ATA CTC ATC ACC TCC TCC TGG 1115
 Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp
 305 310 315
 ATT CTC TCT CCC CCC CAC TCC TTC CAG GAG AGG TTT CCG CCC CAC CAC 1163
 Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His
 320 325 330
 CTG ACG GTG ATC TTG CGC AGA ACA TAC CGG GTG GTC CCT CGC GAG GAG 1211
 Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu
 335 340 345
 GAG CAG AAA TTT GAA GTC GAA AAA TAC ATT GTC CAT AAG GAA TTC GAT 1259
 Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp
 350 355 360
 GAT GAC ACT TAC GAC AAT GAC ATT CGC CTG CTG CAG CTG AAA TCG GAT 1307
 Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp
 365 370 375 380
 TCG TCC CGC TGT CGC CAG GAG AGC AGC GTG GTC CCT ACT GTG TCC CTT 1355
 Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu

385	390	395
CCC CCG GAG GAC CTG CAG CTG CCG GAC TGG ACG GAG TGT GAG CTC TCC	1403	
Pro Pro Glu Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser		
400	405	410
GGC TAC CGC AAG CAT GAG GCC TTG TCT CCT TTC TAT TCG GAG CGG CTG	1451	
Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu		
415	420	425
AAG GAG CCT CAT GTC AGA CTG TAC CCA TCC ACC CCC TGC ACA TCA CAA	1499	
Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln		
430	435	440
CAT TTA CTT AAC AGA ACA GTC ACC GAC AAC ATG CTG TGT CCT GGA GAC	1547	
His Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp		
445	450	455
ACT CGG AGC CGC GGG CCC CAG GCA AAC TTG CAC GAC GCC TCC CAG CCC	1595	
Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln Gly		
465	470	475
GAT TCG GGA CGC CCC CTG GTG TGT CTG AAC GAT CCC CGC ATG ACT TTG	1643	
Asp Ser Gly Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr Leu		
480	485	490
GTG CGC ATC ATC AGC TGG CGC CTG CGC TGT CGA CAG AAG GAT GTC CCG	1691	
Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro		
495	500	505
CGT GTG TAC ACC AAG GTT ACC AAC TAC CTA GAC TGG ATT CCT GAC AAC	1739	
Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn		
510	515	520
ATG CGA CGG TGACCAAGAA CACCCGACTC CTCAAAACCA AATGAGATCC	1788	
Met Arg Pro		
525	527	
CCCCCTTTCT · TC		1800.

[0079] 配列番号：10

30* 配列の長さ：1068

配列の長さ：36

配列の型：核酸

配列の型：核酸

鎖の数：二本鎖

トポロジー：直鎖状

トポロジー：直鎖状

配列の種類：他の核酸 合成DNA

配列の種類：他の核酸 配列番号7を修飾

配列

配列の特徴

ACTGTTTCCC TCAGACATAT GAGGGTGCCT GCAGAA 36. 存在位置：1～173までのアミノ酸欠損

[0080] 配列番号：11

* 特徴を決定した方法：P

配列		
ATG TCT GAG CGA AAC AGT GAC TGC TAC TTT CGG AAT CGG TCA GCC TAC	48	
Met Ser Glu Gly Asn Ser Asp Cys Tyr Phe Gly Asn Gly Ser Ala Tyr		
1	5	10
CGT CGC ACG CAC ACC CTC ACC GAG TCG GGT CCC TCC TCC CTC CCG TGG	96	
Arg Gly Thr His Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp		
20	25	30
AAT TCC ATG ATC CTG ATA GCC AAG GTT TAC ACA GCA CAG AAC CCC AGT	144	
Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser		
35	40	45
CCC CAG GCA CTG CGC CTG GCC AAA CAT AAT TAC TCC CGG AAT CCT GAT	192	
Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp		
50	55	60

39	40	
CGG GAT GCC AAG CCC TCG TCC CAC GTG CTG AAG AAC CGC AGG CTG ACG	240	
Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr		
65 70 75 80		
TCG GAG TAC TGT GAT GTG CCC TCC TCC ACC TCC CCC CTG AGA CAG	288	
Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln		
85 90 95		
TAC ACC CAG CCT CAG TTT CCC ATC AAA GGA CGG CTC TTC GCC GAC ATC	336	
Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile		
100 105 110		
CCC TCC CAC CCC TCG CAG CCT GCC ATC TTT CCC AAG CAC AGG AGG TCC	384	
Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser		
115 120 125		
CCC GGA GAG CGG TTC CTG TCC CGG CGC ATA CTC ATC AGC TCC TCC TGG	432	
Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp		
130 135 140		
ATT CTC TCT GCC CCC CAC TCC TTC CAG GAG AGG TTT CCG CCC CAC CAC	480	
Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His		
145 150 155 160		
CTG ACG GTG ATC TTG CGC ACA ACA TAC CGG GTG GTC CCT GCC GAG GAG	528	
Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu		
165 170 175		
GAG CAG AAA TTT GAA GTC GAA AAA TAC ATT GTC CAT AAG GAA TTC GAT	576	
Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp		
180 185 190		
GAT GAC ACT TAC GAC AAT GAC ATT CGC CTG CTG CAG CTG AAA TCG GAT	624	
Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp		
195 200 205		
TCG TCC CGC TGT CCC CAG GAG AGC ACC GTG GTC CCC ACT GTG TCC CTT	672	
Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu		
210 215 220		
CCC CCG GAG GAC CTG CAG CTG CCG GAC TGG ACG GAG TGT GAG CTC TCC	720	
Pro Pro Glu Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser		
225 230 235 240		
CGC TAC GCC AAG CAT GAG GCC TTG TCT CCT TTC TAT TCG GAG CGG CTG	768	
Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu		
245 250 255		
AAG GAG GCT CAT GTC AGA CTG TAC CCA TCC ACC CCC TCC ACA TCA CAA	816	
Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln		
260 265 270		
CAT TTA CTT AAC AGA ACA GTC ACC GAC AAC ATG CTG TGT GCT CGA GAC	864	
His Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp		
275 280 285		
ACT CGG AGC CGC CGG CCC CAG GCA AAC TTG CAC GAC GCC TGC CAG CGC	912	
Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln Gly		
290 295 300		
GAT TCG CGA CGC CCC CTG GTG TGT CTG AAC GAT GGC CCC ATG ACT TTG	960	
Asp Ser Gly Gly Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr Leu		
305 310 315 320		
GTG GCC ATC ATC ACC TCG CCC CTG GGC TGT CGA CAG AAG GAT GTC CGC	1008	
Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro		

41

42

325	330	335
CGT GTG TAC ACC AAG GTT ACC AAC TAC CTA GAC TCG ATT CGT GAC AAC	1056	
Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn		
340	345	350
ATG CGA CCG TGA		
Met Arg Pro		
355.		

【0081】配列番号：12

配列の長さ：24

配列の型：核酸

トポロジー：直鎖状

配列の種類：他の核酸 合成DNA

配列

GGCGGACTCT TCGTCCTTGG CAAA 24.

* 配列の型：核酸

鎖の数：二本鎖

10 トポロジー：直鎖状

配列の種類：他の核酸 配列番号9を修飾

配列の特徴

存在位置：1～173までのアミノ酸欠損、298及び299位

アミノ酸置換

特徴を決定した方法：P

【0082】配列番号：13

配列の長さ：1068

*

配列

ATG TCT GAG GGA AAC AGT GAC TGC TAC TTT GGG AAT GGG TCA GCC TAC	48
Met Ser Glu Gly Asn Ser Asp Cys Tyr Phe Gly Asn Gly Ser Ala Tyr	
1 5 10 15	
CGT GCC ACG CAC AGC CTC ACC GAG TCG CGT CCC TCC TGC CTC CCG TGG	96
Arg Gly Thr His Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp	
20 25 30	
AAT TCC ATG ATC CTG ATA GCC AAG GTT TAC ACA GCA CAG AAC CCC AGT	144
Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser	
35 40 45	
GCC CAG GCA CTG CGC CTG CGC AAA CAT AAT TAC TCC CCG AAT CCT GAT	192
Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp	
50 55 60	
CGG GAT GCC AAG CCC TCG TCC CAC GTG CTG AAG AAC CCC AGG CTG ACG	240
Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr	
65 70 75 80	
TGG GAG TAC TGT GAT GTG CCC TCC TCC ACC TCC CGC CTG AGA CAG	288
Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln	
85 90 95	
TAC ACC CAG CCT CAG TTT CCC ATC AAA GGA CGG CTC TTC GCC GAC ATC	336
Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile	
100 105 110	
CCC TCC CAC CCC TGG CAG GCT GCC ATC TTT CCC AAG CAC GAA GAG TCG	384
Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Glu Glu Ser	
115 120 125	
CCC CGA GAG CGG TTC CTG TCC CGG CGC ATA CTC ATC ACC TCC TGC TGG	432
Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp	
130 135 140	
ATT CTC TCT CCC GCC CAC TCC TTC CAG GAG AGG TTT CCG CCC CAC CAC	480
Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His	
145 150 155 160	
CTG ACG GTG ATC TTG CGC AGA ACA TAC CGG GTG GTC CCT CGC GAG GAG	528
Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu	
165 170 175	

43

44

GAG CAG AAA TTT GAA GTC GAA AAA TAC ATT GTC CAT AAG GAA TTC GAT 576
 Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp
 180 185 190
 GAT GAC ACT TAC GAC AAT GAC ATT CCG CTG CTG CAG CTG AAA TCG GAT 624
 Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp
 195 200 205
 TCG TCC CGC TGT GCC CAG GAG AGC ACC GTG GTC CCC ACT GTG TCC CTT 672
 Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu
 210 215 220
 CCC CGG GAG GAC CTG CAG CTC CCG GAC TCG ACC GAG TGT GAG CTC TCC 720
 Pro Pro Glu Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser
 225 230 235 240
 CGC TAC CGC AAG CAT GAG GCG TTG TCT CCT TTC TAT TCG GAG CCG CTG 768
 Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu
 245 250 255
 AAG GAG GCT CAT GTC AGA CTG TAC CCA TCC AGC CCC TGC ACA TCA CAA 816
 Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln
 260 265 270
 CAT TTA CTT AAC AGA ACA GTC ACC GAC AAC ATG CTG TGT CCT GCA GAC 864
 His Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp
 275 280 285
 ACT CGG AGC CGC CGG CCC CAG GCA AAC TTG CAC GAC GCC TCC CAG CGC 912
 Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln Gly
 290 295 300
 GAT TCG CGA CGC CCC CTG GTG TGT CTG AAC GAT GCC CGC ATG ACT TTG 960
 Asp Ser Gly Gly Pro Gln Ala Asn Leu Asp Gly Arg Met Thr Leu
 305 310 315 320
 GTG GCC ATC ATC AGC TCG CGC CTG CGC TGT CGA CAG AAG GAT GTC CCG 1008
 Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro
 325 330 335
 CGT GTG TAC ACC AAG GTT ACC AAC TAC CTA GAC TCG ATT CGT GAC AAC 1056
 Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn
 340 345 350
 ATG CGA CGG TCA
 Met Arg Pro
 355。

【0083】配列番号：14

配列の長さ：8

配列の型：アミノ酸

トポロジー：直鎖状

配列の種類：ペプチド

フラグメント型：N末端フラグメント

■

Gly His Arg Pro Leu Asp Lys Cys

5。

【0084】配列番号：15

配列の長さ：12

配列の型：アミノ酸

トポロジー：直鎖状

配列の種類：ペプチド

フラグメント型：中間部フラグメント

配列

Phe Phe Ser Ala Arg Gly His Arg Pro Leu Asp Lys

40 5 10。

【図面の簡単な説明】

【図1】tPAおよびtPAムテインの構造を示す模式図である。

【図2】IgGの構造を示す模式図である。

【図3】参考例1で得られたヒトtPA cDNAの塩基配列およびそれから推定されるアミノ酸配列である。

【図4】プラスミドpTB920の構築図である。

【図5】プラスミドpTB927の構築図である。

【図6】ヒトtPAムテインtPA-1を得るに当って行わされた特定部位変異の模式図である。

【図7】ヒトtPAムテインtPA-1の塩基配列およびそれから推定されるアミノ酸配列である。

【図8】プラスミドpTB1128および1133の構築図である。

【図9】プラスミドpTB1038および1277の構築図である。

【図10】ヒトtPAムテインtPA-6'の塩基配列およびそれから推定されるアミノ酸配列である。

【図11】実施例1で得られた抗ヒト・フィブリノーゲンに対する抗体希釈曲線を表す。フィブリノーゲンに対する反応性(○:参考例5に記載のEIA)およびフィブリンに対する反応性(●:参考例1に記載のEIA)を示す。

【図12】実施例2-(4)に記載の抗フィブリノーゲン・抗tPAムテインbsMoAb(TAF1-42, TAF1-79およびTAF1-228)の精製結果を表す。すなわち、bsMoAbを含有する腹水液より塩析処理でIgG画分を取得し、さらにフィブリノーゲン末端ペプチド結合カラムで精製したのち、ヒドロキシ・アバタイト・カラムに供した結果を示す。クロマト・パターン*20

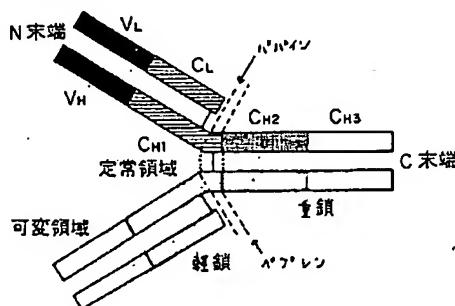
*は溶出液の280nmでの吸光度で示し、矢印は参考例6に記載のEIAでbsMoAb活性陽性のピークを示す。

【図13】実施例2-④に記載の抗フィブリノーゲン・抗tPAムテインbsMoAb TAF1-228をin vitro血漿凝塊溶解試験に供した時の結果を表す。縦軸は非修飾tPA単剤(●)もしくはtPAムテインtPA-6'単剤(○)の溶解能を100%とした時の、それぞれtPA/抗体複合体(●)もしくはtPA-6'/抗体複合体(○)の溶解能を表す(実施例5参照)。

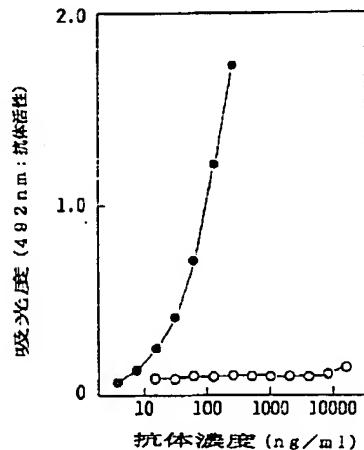
【図14】実施例3に記載の抗フィブリノーゲン・抗tPAムテインbsMoAb FT2-14(●)およびそのF(ab')₂画分(○)を、参考例6に記載のEIAで測定した結果を表す。

【図15】実施例6-③に記載の抗活性化血小板-抗tPAムテイン二重特異性抗体F(ab')₂の活性を参考例20に記載のEIAで測定した結果を表す(実施例6-④参照)

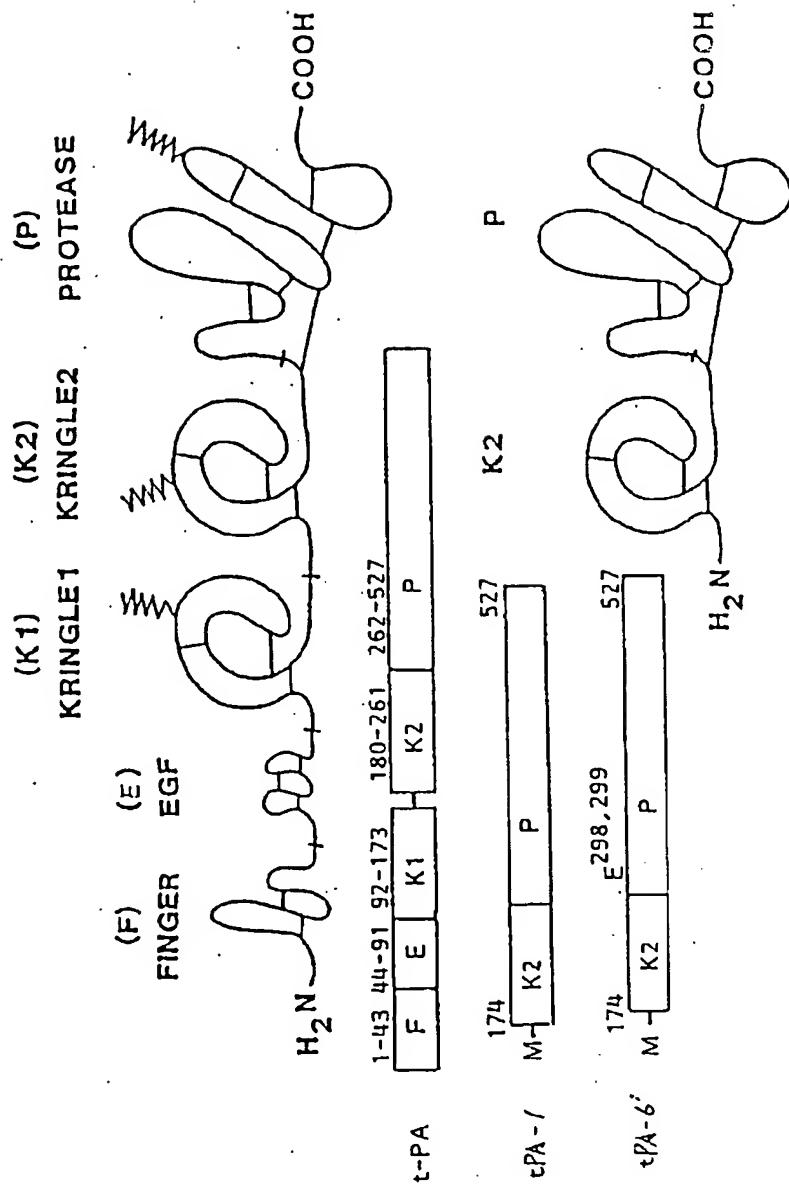
【図2】



【図11】



【図1】



【図6】

特定部位変異の模式図
site directed mutagenesis

t-PA: 5' - E F C S T P A M 174 C G G N S D
GAG TTC TGC AGC ACC CCT GCC TCT GAG GGA AAC ACT GAC - 3'
Primer: 3' - AAG ACG TCG TGG GGA GTA TAC GTA GGA CCT CTC AGA CTC GTA TAC TTG TCA - 5'
NdeI

【図3-1】

AAAACCTCTGCGAGAAAGGGAAAGGGCAAGGGTGAATTAAAGGGACGGCTGTGAAGCAA
 10 20 30 40 50 60
 TCAATGGATGCAATGAAAGGAGGGCTCTGCTGTGCTGGCTGCTGGACCAACTCTTCG
 M D A M K R Q L C V L L C G A V -17
 -35
 TTTCAAGCCAGGAAATCCATGCCATTAGAAAGGGAGGGAGGGAGGGAGGGAGGGAGGG
 V V S P S Q E I H A R R F R G A R S T Y Q V 4
 -1 +1
 TCTGGCAAGGATGAAAAAAAGGAGATGATGATGATGATGATGATGATGATGATGATG
 I C R D E K T Q M I Y Q H Q S W L R P 24
 250 260 270 280 290 300 310 320 330 340 350 360
 TGCTCAAGCAAGGCAACCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
 V V L R S N R V E Y C W C N S G R A Q C H 44
 43 44
 CAGTGCCTGTCAAAAGTTGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGG
 S V P V K S C S E P R C F N G G T C Q Q 64
 370 380 390 400 410 420
 CCCTGATCTCAGATTTCGGTATGCCAGTACCCCCGAGGGATTGGCTGGAAAGTGGCTGG
 A L Y F S D P V C Q C P E Q F A Q K C C 84
 430 440 450 460 470 480
 AAATACATACCAAGGGCCACGGTGTACGGACCCAGGGCATCAGCTACAGCTACAGCTAC
 E I D T R A T C Y E D Q G I S Y R G F W W 104
 91
 490 500 510 520 530 540
 GCACAGGGAGAAGCTGGCAACTGGAAACCAACTGGAAACCAACTGGCAAGGGGGGGGG
 S T P A E S G A E C T N S A L A Q K 124
 550 560 570 580 590 600
 CCTTACACAGGGTGGGAGGGCCAGACGGCCATCAGGCTGGGGGGGGGGGGGGGGGG
 P Y S W R P D A I R L G N H N Y C 144

【図3-2】

610	620	630	640	650	660
GAAACCCAGATCGAGACTCAAAGCCCTGGCTACGTCTTAAGGTACAGCT					
R N P D R S K P W C Y V F K A G K Y S	164				
670	680	690	700	710	720
CAGAGTTCTGGCAGCACCCCTGGCTCTGAGGGAAACAGTGACTGGCTACTTGGCAATG					
S E F C S T P A C S E G N S D C Y F G N	184				
730	740	750	760	770	780
GGGTCACCCCTACCGGGCACAGGCCACAGCCTCACCGAGTCGGGTGGCTCCCTGGGA					
G S A Y R G T H S L T E S G A C L S C	204				
790	800	810	820	830	840
ATTCCATGATCCCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGGCCAGGCACCTGG					
N S M I L G K V Y T A Q N P S A Q A L	224				
850	860	870	880	890	900
GCCCTGGGGAAACATAATTACTGCCGGAAATCCTGGATGGGATGCCAAAGCCCTGGGTGCCACCG					
G L G K H N Y C R N P D G D A K P W C H	244				
910	920	930	940	950	960
TGCTGAAGAACCCGGAGCTGACGTGGGACTGTGATGTCCTCCACCTGCC					
V L K N R L T W E Y C D V P S C S T C	264				
970	980	990	1000	1010	1020
GCCTCTGAGACAGTACAGGCCAGGCTCAGTITCGCATCAAGGGAGGGCTCTGGCCGACATCG					
G L R Q S Q P F R I K G G L F A D I	284				
1030	1040	1050	1060	1070	1080
CCTCTCCACCCCTGGCCAGGGCTGGCATCTTGGCCAAGCCACAGGAGGGTGGCCGGAGGGGT					
A S H P W Q A A I F A K H R R S P G E R	304				
1090	1100	1110	1120	1130	1140
TCCCTGTGGGGAGGATCATCTAGCTGGATTCCTCTGCTGCTGCTGCTGCTTCC					
F L C G I L S C W I L S A A H C F	324				
1150	1160	1170	1180	1190	1200
AGGAGAGGGTTCTGGGACCCGACCTGACGGTGTATCTGGGAGAACATACCGGGTGGTCC					
Q E R F P H H L T V I L G R T Y R V V	344				

[図3-3]

1210 1220 1230 1240 1250 1260
 CTGGCCAGGGAGGAGAAATTGAAAGTCGAAAGAACATTGTCCTAAAGGAATTCCGATG
 P G E E Q K F E V E K Y I V H K E F D 364

 1270 1280 1290 1300 1310 1320
 ATGACACTTACGGACAAATGGACATTGGCGCTGGCAATCGGATTGGTCCCCCTGCTG
 D D T Y D N D I A L L Q L K S D S R C 384

 1330 1340 1350 1360 1370 1380
 CCCAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
 A Q E S S V V R T V C L P E D L Q L P 404

 1390 1400 1410 1420 1430 1440
 ACTGGACGGAGTGGAGCTCTCCGGCTAACGGCAAGGATGGGCTCTCTCTATT
 D W T E C F L S G Y G K H E A L S P F Y 424

 1450 1460 1470 1480 1490 1500
 CGGAGCCGGCTGAAGGGGGCTCATGTCAGGACTGTACCCATTCAGGGACATCACAC
 S E R L K E A H V R L Y P S R C T S Q 444

 1510 1520 1530 1540 1550 1560
 ATTACTAACGAAACAGTCACCGACAAACATGGCTGTTGGAGAGACACTCCGGCC
 H L N R T V T D N M L C A G D T R S G 464

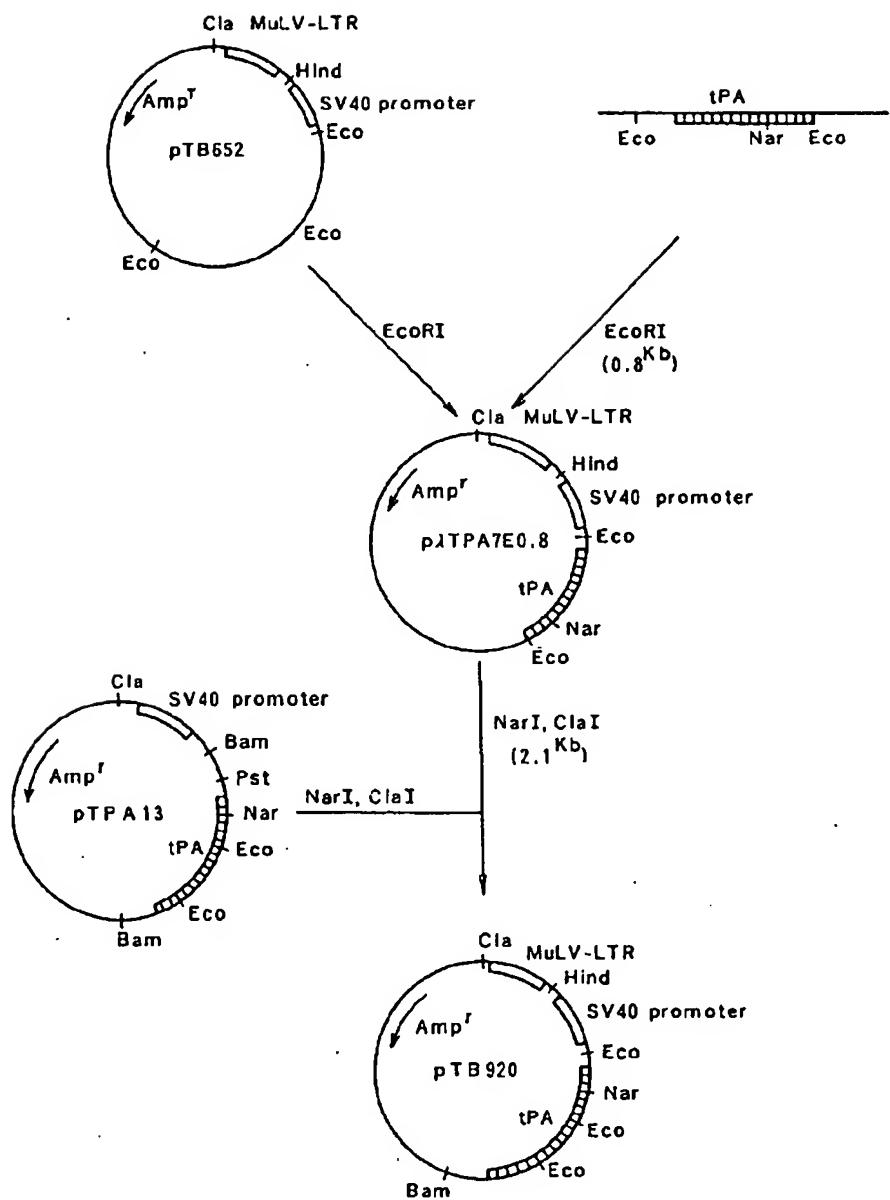
 1570 1580 1590 1600 1610 1620
 GGCCTCCAGGCAAACCTGGCACGCCCTGGAGGGGATTCAGGGAGGGCCCTGGGTG
 G P Q A N L H D A C Q G D S G P L V C 484

 1630 1640 1650 1660 1670 1680
 TGAAACGATGGCCGCATGACTTTGATAAACATCATAGCTGGGGCTGGCTGGGGAG
 L N D G R M T L V G I S W G L G C G Q 504

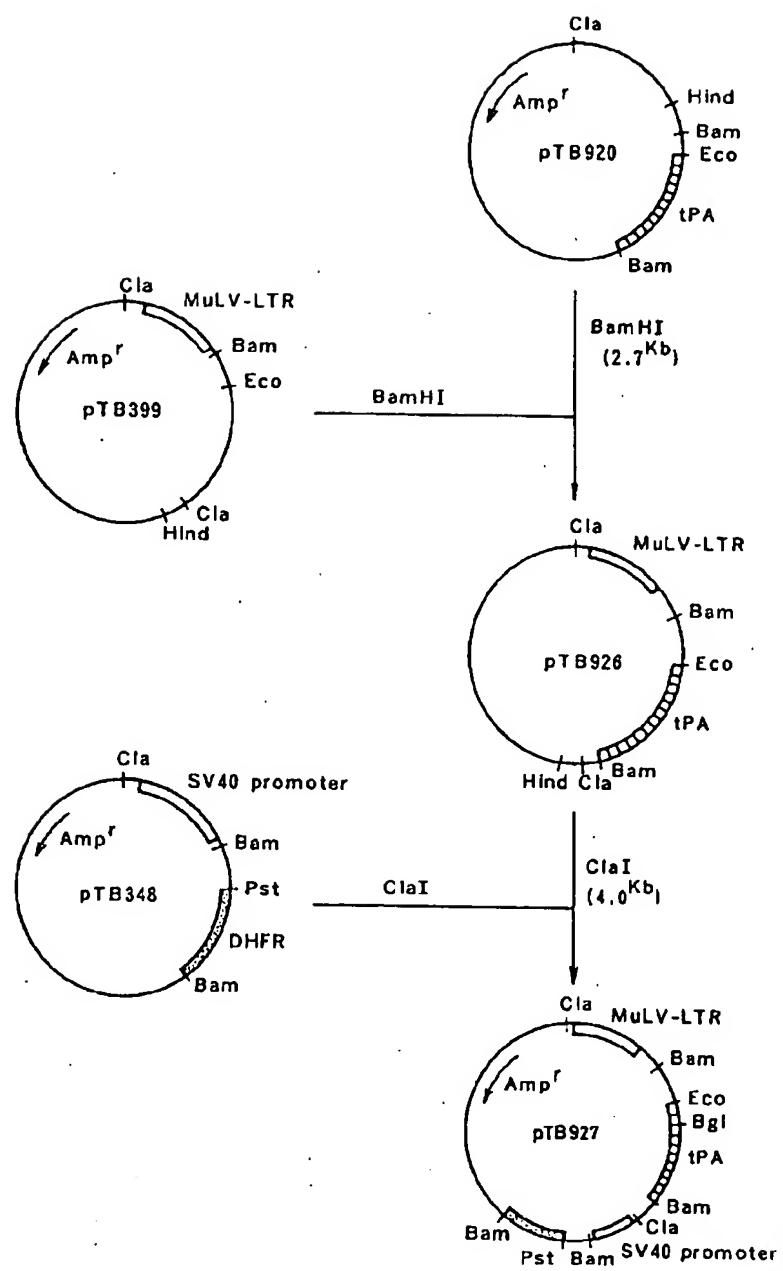
 1690 1700 1710 1720 1730 1740
 AGGATGTCCTGGTGTGACACCAAGGTTACCAACTACCTAGACTGGATTCTGAG
 K D V P G V Y T K V T N L D W 1 R D N 524

 1750 1760 1770 1780 1790 1800
 TGGGACCGTGACCCAGGAACACCCGACTCCTCAAAAGCAAATGAGATCCGGCT
 M R P 527

【図4】



【図5】



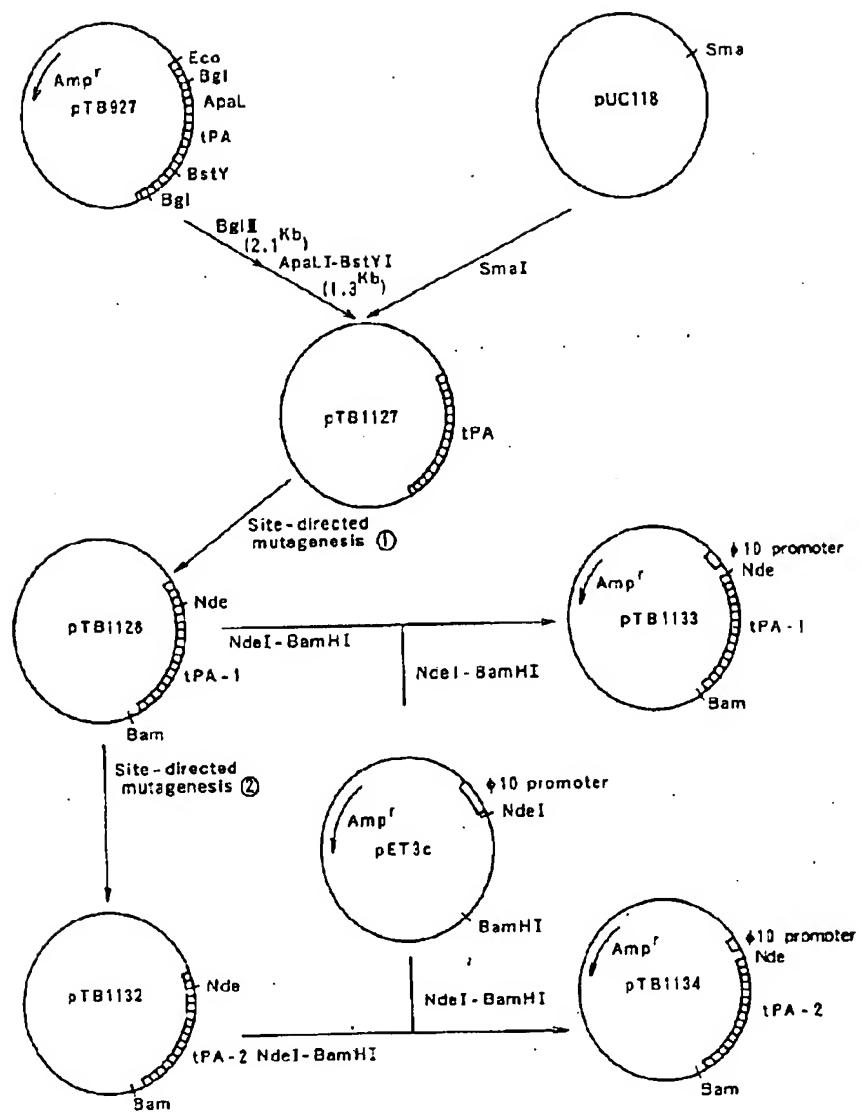
【図7-1】

ATGTCCTGAAAGGAAACAGTGTACTGCTACTTGGAAATGGGT	30	40	50	60
M S E N S D	C Y F G N G	S A Y R G T	C A G G C A C C G C A C	T G G G C A C C G C A C
S L T E S G	S C L P W N	S M I L G K	I G K	I 120
AGCCTCACCGAGTGGGTGCTCCCTGCCCTGGGAATTCATGATG	90	100	110	120
Y T A Q N P S D	A Q S A P W C	A L G L V	N Y C	G A T A G G C A A G
GTTACACAGCACAGAACCCAGTGGCCACATGGCACTGGG	140	150	160	180
R N P D G D	R N P D G D	A K P W C H	K H N Y	G C A C T G G G C A A C
CGGAATCTGATGGGGATGCCAAGGCCCTGGTGGCCACATG	200	210	220	230
W E Y C D V	W E Y C D V	S C S T C G	Q L R Q	T G G A C A G T A C G G C T G A C
TGGGGAGTACTGTGATGTCGGCTGGCTGGCTGGCTGGCT	260	270	280	290
Q F R I K G G	Q F R I K G G	S P L F A D	S H P W	Q S Y S Q P
CAGTTTGGCATCAAAGGAGGGCTCTGGGGACATGGGCTT	310	320	330	340
A T C T T I F A R R	A T C T T I F A R R	R R S P Q D	R R F L C	T G G C A A G G C T G G C
ATCTTGGCAAGCACAGGGAGTGGGGGGAGAGGGGGGGGG	370	380	390	400
I F A K H R R	I F A K H R R	A A H C	G G G	G G G G C A T A C T C A T C
AGCTCCTGGCTGGATTCCTCTGCTTACGGCCCACTGCT	430	440	450	460
S C W I L S	S C W I L S	A A H C F	F Q E R	A G A G G A T T C C G G C C A C C A C
CTGACGGGTGATCTTGGCGAGAACATACCGGGTGGT	490	500	510	520
L T V I L G R T	L T V I L G R T	V V P G	E E E	T G G C G A G G A G G C A G A A T T
GAAGTCGAAAAATACATTGGTCCATAAGGAATTTCGATG	550	560	570	580
E V E K I	E V E K I	H H D D	F P D	A C A T G A C A T T D N

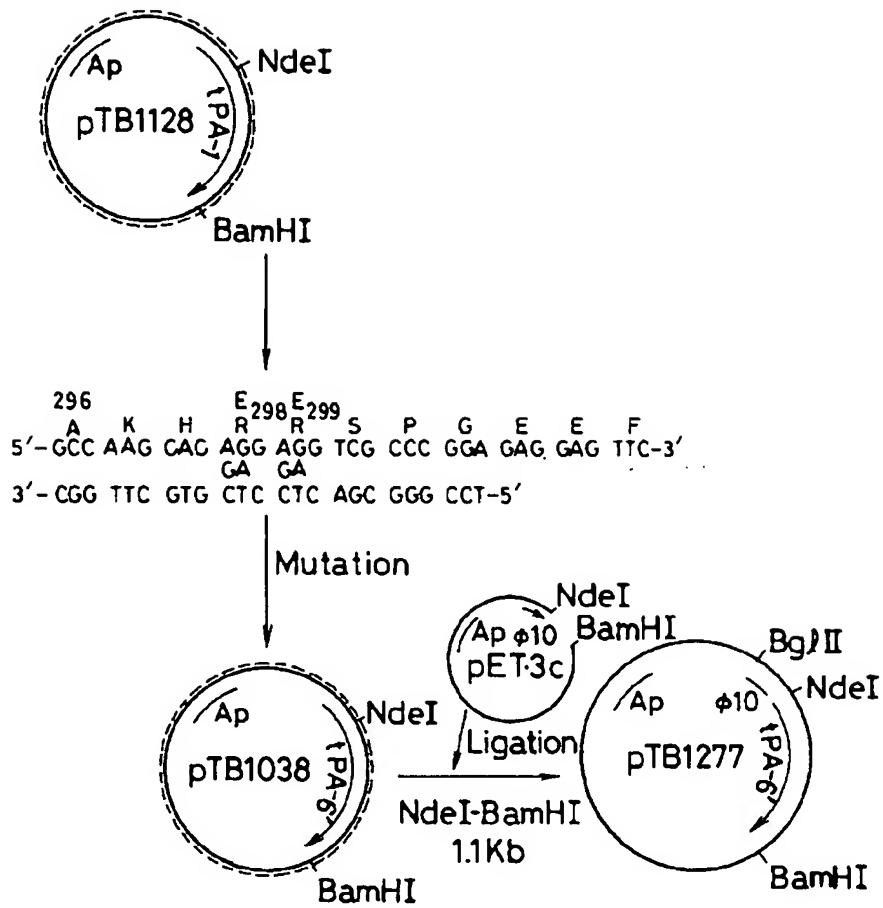
[図7-2]

610 G G G C T G C T G C A G G C A A T C G G A T T C G T C C C G C T G T G C C G A G G C A G G C A C C G T C C G C 660
 A L L Q L K S D S R S R C A Q E S S V R
 620 630 640 650
 670 A C T G T G C C T T C C C C G G A G G C A C C T G C A G G C T G C C G G A C T T G G A C G G A C T G A G C T C T C C 720
 T V C L P P E D L Q L P D W T E C E L S
 680 690 700 710
 730 G C C T A C G G C A A G C A T G A G G C C T T G T C T C T A T T C G G A G G G G C T G A A G G A G G C T C A T 760
 G Y G H E A L P F S E R L K E A H
 740 750 760 770
 790 G T C A G A C T G T A C C C A T C C A G G C C T G C A C A T C A C A C A T T T A C T T A A C A G A A C A G T C A C C 840
 V R L P Y S R C T S Q H T L L N R T V T
 800 810 820 830
 850 A C A A C A T G C T G T G T A C T G A C A C T G A G G C G G A G G C A A C T T G C A C C A C 900
 D N M L C A G D T R S G C P Q A N L H D
 860 870 880 890
 910 G C C T G C C A G G C C A T T C G G A A G G C C C C T G T G T G T G C T G A C T T G A C T T G 950 960
 A C Q D S G G P L V C L N D G R M T L
 920 930 940 950
 970 G T G G C A T C A G G C T G G G C C T G G G C T G G A C A G G A A G G A T G T C C P G V Y
 V G I S W G L G Q K D V P G V Y
 980 990 1000 1010 1020
 1030 A A G G T T A C C A A C T A C C T A G A C T G G A T T C G T G A C A A C A T G C G G A C C G T G A C A C C
 K V T N D W I R D N M R P

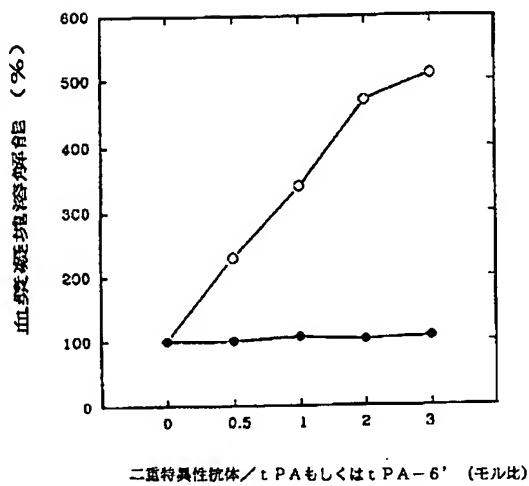
【図8】



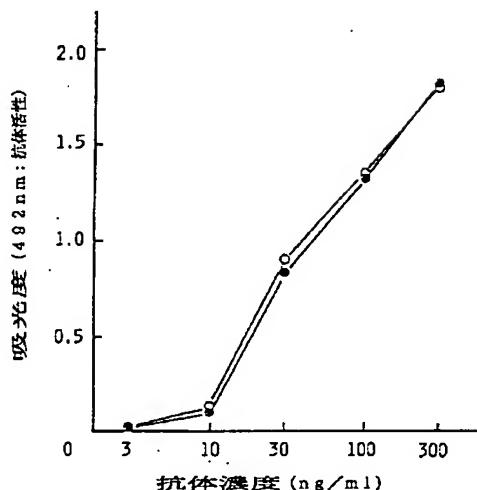
【図9】



【図13】



【図14】



〔図10-1〕

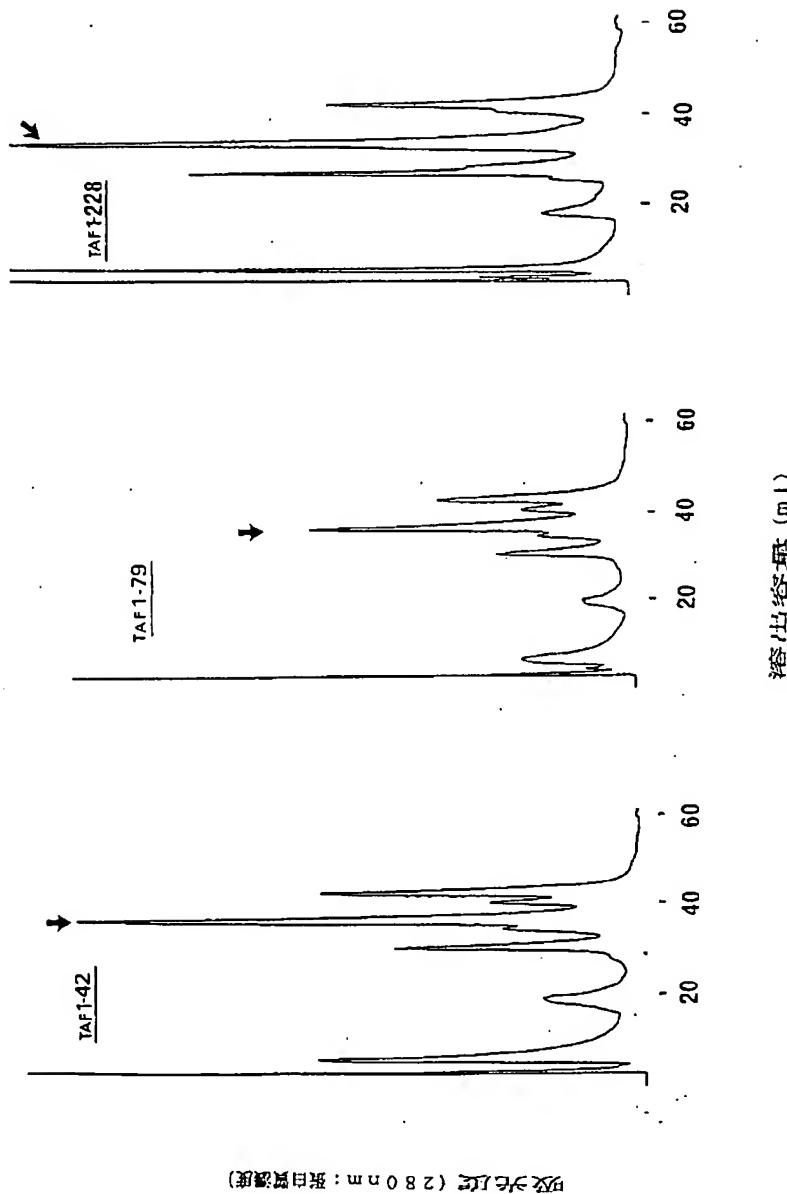
ATGTCTGAGGAAACAGG_N
 1_S 0_E 2_D 0_F 3_V 0_T 4_G 0_C 5_A 0_G 6_H
 173

AGCCTCAC_T
 7_S 0_E 8_D 0_F 9_V 0_T 10_G 0_C 11_A 0_G 12_H
 13_S 0_E 14_D 0_F 15_V 0_T 16_G 0_C 17_A 0_G 18_H
 19_S 0_E 20_D 0_F 21_V 0_T 22_G 0_C 23_A 0_G 24_H
 25_S 0_E 26_D 0_F 27_V 0_T 28_G 0_C 29_A 0_G 30_H
 31_S 0_E 32_D 0_F 33_V 0_T 34_G 0_C 35_A 0_G 36_H
 37_S 0_E 38_D 0_F 39_V 0_T 40_G 0_C 41_A 0_G 42_H
 43_S 0_E 44_D 0_F 45_V 0_T 46_G 0_C 47_A 0_G 48_H
 49_S 0_E 50_D 0_F 51_V 0_T 52_G 0_C 53_A 0_G 54_H
 55_S 0_E 56_D 0_F 57_V 0_T 58_G 0_C 59_A 0_G 60_H

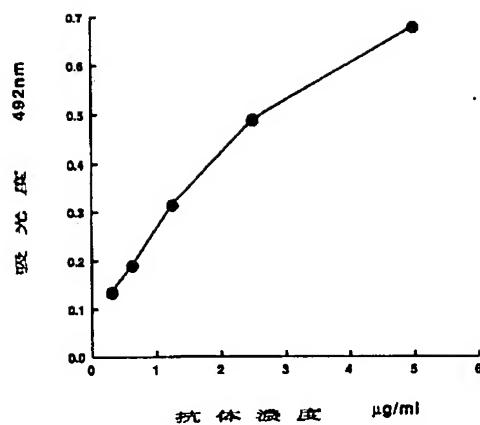
[図10-2]

GCGCTGCC_L⁶¹⁰QACCTGAA_K⁶²⁰ATCGGATT_S⁶³⁰CGCTGG_C⁶⁴⁰CAAGGAGG_S⁶⁵⁰CGTGG_V⁶⁶⁰CC_R
 ATGTC_V⁶⁷⁰GTGCC_L⁶⁸⁰GGACCC_P⁶⁹⁰GGCTGCC_P⁷⁰⁰GGAGG_T⁷¹⁰GTGAG_E⁷²⁰CTC_S
 GCGCTGCC_L⁷³⁰AAACCATGAA_H⁷⁴⁰GCTTGT_S⁷⁵⁰CTCTAT_V⁷⁶⁰GGAGCC_R⁷⁷⁰GAAGGAGG_A⁷⁸⁰CTCA_H
 GTCAGAC_R⁷⁹⁰GTACCC_P⁸⁰⁰ATGCC_S⁸¹⁰CAAC_Q⁸²⁰ATGCC_R⁸³⁰TAA_C⁸⁴⁰AGTC_A_T
 GACAACATGCC_M⁸⁵⁰LTGTGCC_A⁸⁶⁰AGACACT_D⁸⁷⁰GGAGC_A⁸⁸⁰CCAGGCC_S⁸⁹⁰AACTTGC_A⁹⁰⁰CGAC
 GCGCTGCC_C⁹¹⁰GGCGGATT_S⁹²⁰GGAGGCC_P⁹³⁰GGCTGTC_V⁹⁴⁰GTGAG_D⁹⁵⁰CCATGAC_T⁹⁶⁰CT_L
 GTCGGCA_V⁹⁷⁰CATCAC_S⁹⁸⁰GGCTGG_G⁹⁹⁰GGACAGAA_K¹⁰⁰⁰GGATGTCC_P¹⁰¹⁰GGTGT_V¹⁰²⁰CA_T
 AACGCTTA_K¹⁰³⁰CAACTAAC_T¹⁰⁴⁰TAGACTGGAT_D¹⁰⁵⁰TGGATGACAAC_N¹⁰⁶⁰ATGCC_R¹⁰⁷⁰GGACCGTGA_P

〔図12〕



【図15】



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